Universidade de Aveiro Departamento de Química
Ano 2017

Andreia Patrícia Macedo Fernandes

Separação de enantiómeros de ácido mandélico usando sistemas aquosos bifásicos contendo seletores quirais

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo Métodos Biomoleculares, realizada sob a orientação científica do Doutor João Manuel Costa Araújo Pereira Coutinho, Professor Catedrático do Departamento de Química da Universidade de Aveiro, CICECO, e da Doutora Sónia Patrícia Marques Ventura, Equiparada a Investigadora Auxiliar do Departamento de Química da Universidade de Aveiro, CICECO.

This work was partly funded by FCT project SAICTPAC/0040/2015.

“Don’t be afraid of hard work. Nothing worthwile comes easily”
- Gertrude Elion

o júri

presidente

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agradecimentos

Inicialmente um grande obrigado ao Professor João Coutinho e a Drª a Sónia Ventura pela oportunidade que me deram de desenvolver este tema de dissertação. Obrigado a Francisca, Matheus e Tânia por toda a disponibilidade, preocupação e conselhos que me deram durante esta última fase académica. Ao grupo do PATh que acolheu durante um ano. Um obrigado a Liliana, Marta, Margarida e Vanessa pelo apoio moral e carinho que me deram. Agradecer as migas: Ana Cristina, Ana Rufino, Carmen, Cristina, Diana, Inês, Nicole, Rita e Tânia por 5 anos de grandes loucuras. Quero agradecer as minhas 3 pequenas pedaças: Inês, Pipa e Catarina, por terem feito os últimos 3 anos terem um sabor especial. À minha prima Lili que entrou comigo nesta aventura. Aos meus primos e ao meu afilhado, que estiveram presentes ao longo desta caminhada. Ao meu irmão Nuno pelo apoio. Mas o maior obrigado para os meus pais que permitiram e incentivaram a que eu entrasse nesta loucura de 5 anos, com todo o amor, apoio e carinho que só eles me souberam dar.

palavras-chave

Enantioseparação, sistema aquoso bifásico, seletor quiral, proteína, sacarose, ácido mandélico, docagem molecular

resumo

A quiralidade é a uma propriedade importante na indústria farmacêutica, uma vez que um enantiómero de um fármaco pode exercer o efeito terapêutico desejado enquanto o outro pode ser inerte ou mesmo nefasto. Embora vários fármacos sejam comercializados na sua forma racémica, as entidades regulatórias aconselham o desenvolvimento de fármacos enantiomericamente puros e mais seguros. Neste contexto, a indústria farmacêutica procura formas baratas e eficientes de produzir fármacos enantiomericamente puros, sendo este o objetivo da presente tese.

A separação enantiomérica do ácido mandélico (AM), aqui utilizado como um fármaco racémico modelo, será tentada recorrendo a sistemas aquosos bifásicos (SABs) constituídos por seletores quirais de origem natural (proteínas e açúcares). Serão usadas duas abordagens: (i) a introdução de proteínas como seletores quirais em diferentes tipos de SABs; e (ii) o uso de (D)-sacarose simultaneamente como seletor quiral e componente de fase em SABs. Na primeira abordagem, foram utilizados diferentes tipos de SABs (polímero+polímero, polímero+sal, sal+líquido iónico (LI), polímero+LI e polímero+açúcar) e duas proteínas (albumina de soro bovino – BSA – e citocromo C – Cit c). A escolha das proteínas assentou em resultados de molecular docking que indicaram interações distintas entre diferentes proteínas e os enantiómeros do AM. Nestas fases, os sistemas constituídos por PPG400+(D)-Sacarose+BSA (excesso enantiómerico de -5.9± 0.5%) e PPG400+dihidrogeno fosfato de colínio+Cit c (excesso enantiomérico de -9.0 ± 1.2%) revelaram-se os mais eficientes. As proteínas e os constituintes de fase dos SABs afetaram a separação enantiomérica de ácido mandélico. Uma vez que a docagem molecular não considera as interações com os componentes de fase, esta abordagem revelou ser incapaz de prever o desempenho das proteínas como seletores quirais em SABs.

Com o objetivo de ultrapassar as limitações de seletividade enantiomérica e melhorar a simplicidade operacional da tecnologia proposta, a (D)-sacarose foi usada simultaneamente como formador de fase e seletor quiral em SABs. Depois de uma otimização cuidada, foi possível obter um excesso enantiomérico máximo de -12.3 ± 0.5% com um SAB constituído por polímero e (D)-sacarose.

keywords

Enantioseparation, aqueous biphasic systems, chiral selector, protein, sucrose, mandelic acid, molecular docking

abstract

Chirality is an important property for the pharmaceutical industry, since one enantiomer of a drug can exert a therapeutic action, while the other may be inert or even nefarious. While several drugs are commercialized as racemates, regulatory bodies strongly encourage the development of safer enantiopure drugs. In this context, pharmaceutical industry seeks for cheap and efficient ways of obtaining enantiopure pharmaceuticals and this is the main objective of this thesis.

The enantiomeric separation of mandelic acid (MA), here used as a model racemic drug, using aqueous biphasic systems (ABS) composed of natural chiral selectors (proteins and sugars) will be proposed. Two different approaches were used: (i) the introduction of proteins as chiral selectors in several types of ABS; and (ii) ABS formed by D-Sucrose as both phase former and chiral selector. Within the first approach, different types of systems (polymer+polymer, polymer+salt, polymer+sugar, and ionic liquids (ILs)+salt, ILs+polymer) and of proteins (bovine serum albumin –BSA - and cytochrome C – Cyt C) were used. These two proteins were chosen based on molecular docking results that shown distinctive interactions with the two MA enantiomers among eleven screened proteins. PPG400+(D)-sucrose+BSA system (enantiomeric excess of -5.9 ± 0.5%) and PPG+cholinium dihydrogenphosphate+Cyt C (enantiomeric excess of -9.0 ± 1.2% were the most efficient ABS developed up to this stage. Both the protein and ABS phase formers affected the enantioseparation of MA. Since molecular docking does not encompass the interactions with the ABS phase formers, it was limited at predicting the proteins' performance as chiral selectors in ABS.

In order to surpass the limited enantioselectivity displayed and to improve the operational simplicity of the proposed technology, (D)-sucrose was employed as both chiral selector and phase former in ABS. After a proper optimization, it was possible to achieve a maximum enantiomeric excess of -12.3 ± 0.5% with an ABS composed of polymer and (D)-sucrose.

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List of abbreviations

ABS – Aqueous biphasic system

ADT – Auto DockTools

BSA – Bovine serum albumin

CCC – Counter current chromatography

CE – Capillary electrophoresis

CD - Cyclodextrin

CSP – Chiral stationary phases

Cyt C – Cytochrome C

DAD – Diode array detector

Dex - Dextran

DFT – Density functional theory

ELLE – Enantioselective liquid-liquid extraction

e.e. – Enantiomeric excess

E.E. – Extraction efficiency

HPLC– High performance liquid chromatography

HP-β-CD – Hydroxypropyl-β-cyclodextrin

IL – Ionic liquid

MA – Mandelic acid

MD – Molecular docking

PEG – Polyethylene glycol

PPG – polypropylene glycol

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1.Introduction

Chirality is a property of a molecule that is non-superimposable with its mirror image, each image being an enantiomer, as it happens with the mirror images of human hands – Figure 1. [1] Chirality is a crucial property that nature uses to perform its functions, such as protein folding, DNA double helix, phospholipid membranes and photosystems.[2]

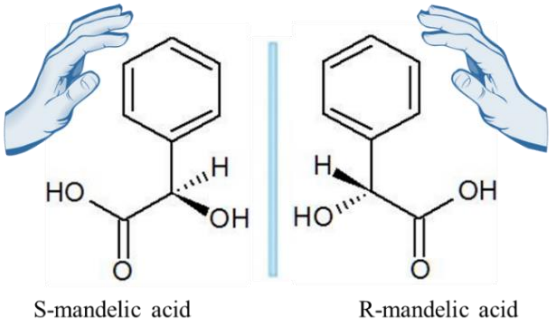

Figure 1 - Enantiomers of mandelic acid as a representative example of chirality with the analogy to human hands.

Stereoisomers are molecules that have an identical atomic constitution and bonding,[3] but differ in the three-dimensional arrangement of their atoms. Despite having the same chemical structure and physical properties (in most cases excepting for optical activity), a pair of enantiomers may induce very different effects in living systems. Differences at the level of pharmacological, toxicological, metabolic, among other biological activities, may occur. Hence, chirality occupies an important role in many fields, such as in agrochemical industry,[4] food safety,[5] and, especially, pharmaceutical industry.[6] Here, an enantiomer may cause the desired therapeutic effect (eutomer), and the other (distomer) may be less potent, bio-inert or even toxic.[7,8] Active pharmaceutical ingredients like the non-steroidal anti-inflammatory drug ibuprofen and the anticoagulant agent warfarin have their enantiomers with the same activity and are taken as racemates in a safe way. Other racemic drugs have one of the enantiomers that causes harmful side effects.[9] The most popular case occurred in the 1950s, when the immunomodulatory agent thalidomide was prescribed for insomnia' and cutaneous diseases' treatment,[10] as a sedative, and mainly to heal nausea in pregnant women.[11] While this drug was being marketed as a racemate, the unknown teratogenic action of the *S* enantiomer caused anomalies in children, such congenital heart diseases, malformations in the members and ocular abnormalities.[12] Other example is methadone which is used to treat chronic pain,[13] but the *S*

enantiomer is linked to cardiac arrhythmia due to its interaction with the human Ether-à-go-go Related Gene (hERG) channels in cardiac myocites. In addition, Parkinsons' disease symptoms are treated with levodopa, however the enantiomer D causes granulocytopenia. In this context, regulatory entities perform a tight control of the medicines going to be commercialized.[9,14] Although the use of enantiopure drugs is preferred, if the racemic form is wanted by the industry, the supply of the detailed pharmacologic, pharmacokinetic and toxicological data for each enantiomer and respective racemate is mandatory. This calls the attention for the development of efficient approaches to obtain pure enantiomers.[15]

In this context, this work intends to develop an alternative method for chiral resolution. For that purpose, aqueous biphasic systems (ABS) with proteins acting as chiral selectors will be studied. This alternative process is intended to become an easy, flexible, and environmentally friendly alternative to the techniques used today.

1.1. Available approaches to produce pure enantiomers

Two distinct approaches are used to obtain enantiopure compounds, as shown in Figure 2, namely (i) the chiral approach in which the synthesis of the desired enantiomeric molecule is focused, and (ii) the racemic approach describing the separation of enantiomers of a racemic mixture.[16]

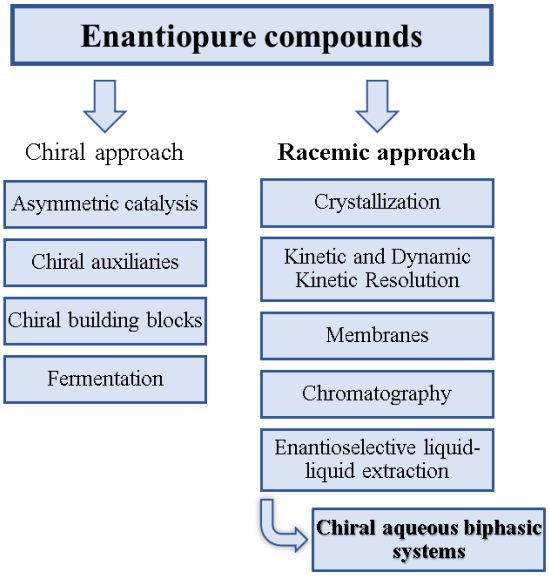

Figure 2 - Summary of different methods used to produce enantiopure compounds. (Adapted from [1])

The chiral approach (or asymmetric synthesis) consists on the enantioselective conversion of an achiral molecule into a chiral one or on the diastereoseletive

conversion of a chiral molecule into other chiral compound. [17] This is the preferred and most robust approach to produce enantiopure compounds and it covers four main different methods according to Figure 2. Asymmetric catalysis is based on small amounts of enantiopure catalysts that can promote desired reactions and lead to the formation of large amounts of enantiopure products.[18] Biocatalysts,[19] metal-ligand complexes derived from chiral ligands[20] and chiral organocatalysts[21] are the chiral catalysts most employed. Chiral auxiliaries are organic compounds that bond to the starting material to form diastereomic species by enantioselective reactions.[22] The formation of these species implies an energetic difference between them, allowing their enantiodiscrimination. Chiral building blocks for synthesis of enantiopure molecules can also be employed, as shown by Magdziak and collaborators.[23] The chiral starting materials are transformed into the desired enantiopure compound by multiple and consecutive reactions; this is the least complicated way. Finally, the selective natural metabolism of microorganisms may also be used to produce optically pure compounds *via* fermentation as done already at industrial scale to obtain enantiopure amino acids.[24] In general, the methods of the chiral approach are highly efficient, but require large stoichiometric amounts of enantiopure raw material and usually need numerous, time consuming and laborious reactions, being expensive and complex to operate.

The racemic approach (or enantioseparation) appears as simpler and more flexible candidate to substitute the asymmetric synthesis.[25] An initial asymmetry is usually needed for enantioseparation, often by the introduction of specific chiral partners, such as reactants, solvents, carriers or solid surfaces.[26] Enantioseparation can be easily achieved with at least one of the commercially available chiral selectors. Many chiral selectors exist and can be obtained from natural sources or from synthetic routes,[27] as shown in Figure 3. Due to their diversity, the identification of good chiral selectors for a specific racemate is usually time consuming and laborious.

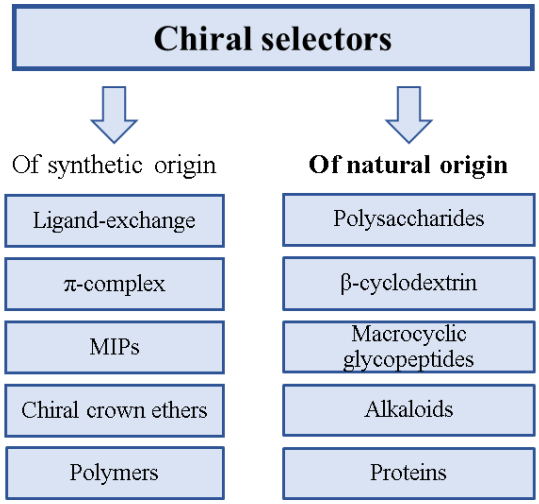

Figure 3 - Summary of the most used chiral selectors organized according to their origin. (Adapted from [28]).

The key in chiral recognition is the formation of diastereoisomeric complexes between the enantiomers and the chiral selector. Molecular recognition occurs because of the differences in the Gibbs free energy between the two diastereoisomeric "enantiomer–chiral selector" complexes. Easson and Stedman[29] proposed that a minimum of three points of attachment is needed for chiral recognition, as shown in Figure 4. Thus, the key for the three-point interaction model is that at least three simultaneous interactions occur with three different substituents attached to the stereogenic center.[30] Two different interactions with the same substituent increase the chiral selector–ligand binding energy and has no effect on the chiral recognition efficiency. It should be noted that three simultaneous interactions are not always required,[31] namely in the case chiral selectors have π-complexes and use aromatic associations that discriminate between two similarly rigid chiral molecules (*i.e.* pseudo-two point interaction model). There are different intermolecular forces that may be taking place between two enantiomers and the chiral selector, namely hydrogen bonding, steric hindrance, π-π stacking, ion-dipole, dipole-dipole, dipole-induced-dipole, van der Waals and electrostatic interactions.[32]

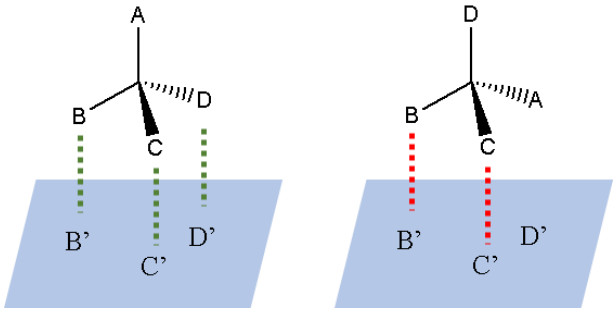

Figure 4 - Graphical representation of the "Three Point Interaction Model". (Adapted from [32]).

The main enantioseparation methods used today are crystallization, chromatography, kinetic and dynamic kinetic resolution and enantioselective liquid-liquid extraction (ELLE) – Figure 2. Since Pasteur's discovery on the crystallization of two enantiomers of sodium ammonium tartrate,[33] crystallization is one of the techniques most adopted by industry. Two major types of enantioselective crystallization exist, namely the commonly designed as classical resolution, where the target racemate is transformed into two diastereomeric salts of distinct solubility by an enantiopure resolving agent, and the preferential crystallization of conglomerates. Despite being easily applicable after other enantioseparation techniques, simple to operate and cost-efficient, crystallization is only an option for few chiral compounds, needs an excessive handling of solids and normally is recognized for its low yields (of around 50%).[34]

The kinetic resolution is a process based on the difference of consumption rates of the two enantiomers in an enantioselective reaction in presence of a chiral catalyst or reagent. From this process results an enantio-enriched sample of less reactive enantiomer.[35] There is a variation of this method called dynamic kinetic resolution, where it is possible to convert the achiral reactant with 100% completion because both enantiomers are in equilibrium.[36] Nevertheless, the absence of suitable reaction partners and the typically high purity standards required for the product limits its application.

Membrane-based enantioselective processes are divided into two main types. One uses a non-enantioselective membrane that assists in an enantioselective process,[37] and the other has enantioselective chiral membranes directly performing the separation.[38] Membrane-aided techniques consist on attractive candidates to enhance the greenness, energy efficiency, easiness and continuous mode operation of the enantioseparation processes.[39] However, there is a need for several operational stages to reach high purities, the relatively low transport rates of the membranes, the risk of membrane fouling or loss of stability for long operation times.[37]

Enantioselective chromatography is not only an useful analytical tool, [40] but also a preparative technique to obtain pure enantiomers from a large diversity of racemates.[41] For enantioseparation, high performance liquid chromatography (HPLC) [42] can be performed using chiral mobile phases or chiral stationary phases (CSP).[43] With this, chromatographic techniques became more reliable, flexible, quick and efficient,[44] delivering both enantiomers with a high optical purity. However,

chromatographic techniques are limited by high losses of the product and limited scalability.[40]

ELLE appears as a more appropriate solution for large scale applications. Conventionally, ELLE combines the concepts of enantiomeric recognition based on the three-point model and solvent extraction using water-immiscible volatile organic solvents.[46,47] The introduction of one chiral selector (e.g. derivatives of crown ether, tartaric acid, cyclodextrin, amino acids, metallic complexes) and its partition between the two immiscible phases will control the enantioseparation,[47] as shown in Figure 5. Monophasic chiral recognition was used by Ren *et al.* [48] The authors proposed the use of L-tartaric acid esters as chiral selectors in the organic phase of an ELLE system for the enantioseparation of racemic ibuprofen. The first step of that work was the study of the chiral recognition mechanism using the density functional theory (DFT). Two carbonyl and two ether groups exist in the L-tartaric acid dihexyl ester and ibuprofen has one carboxyl group, all able to form hydrogen bonds for chiral recognition interactions. Moreover, the two hexyl chains present in the L-tartaric acid dihexyl ester structure and the phenyl group existing in the ibuprofen can induce a steric hindrance, which discriminates between the two ibuprofen enantiomers. Both *R*-ibuprofen and *S*-ibuprofen can form complexes with L-tartaric acid dihexyl ester because both complexes have negative binding energies, with stronger interaction with *R*-ibuprofen. The results show that O−H···O interactions are less than 3.0 Å of distance and greater than 0° of angle, indicating that the chiral recognition mainly involves hydrogen-bonding interactions. The "*R*-ibuprofen-L-tartaric acid dihexyl ester" complex is more stable, because it has a number of hydrogen bonds 2-fold higher than in the "*S*-ibuprofen-L-tartaric acid dihexyl ester" complex. Moreover, biphasic recognition appears in ELLE for better enantioselectivities. That is, the use of two distinct chiral selectors that will be located in opposite phases of the ELLE system. In theory, this is a better alternative since chiral selectors and "chiral selector-enantiomers" complex will be only soluble in one of the phases. An ELLE system formed by D-diisopropyl tartrate (DIPT, hydrophobic chiral selector) in the organic phase and hydroxypropyl-β-cyclodextrin (HP-β-CD, hydrophilic chiral selector) in the aqueous phase was employed to separate racemic amlodipine. DIPT has a preference to *R*-amlodipine, thus concentrating the molecule in the organic phase. The *S*-enantiomer was recognized by HP-β-CD, forming a complex in the aqueous phase. A total separation of amlodipine enantiomers occurs by their partition to opposite phases.[49] Finally, reactive

enantioseparation in ELLE was suggested by Huang *et al.*[50], who showed that it is possible to use a chiral ketone as chiral extractant with Aliquat 336 to separate amino acid racemates. Ketone (*S*)-3, synthetized from (S)-1,1´- bi – 2- naphtol (BINOL) – 3-carboxil, shows high kinetic enantioselectivity for L-amino acids with hydrophobic substituents and a high thermodynamic enantioselectivity for D-amino acids with polar side chains. Reversible ketamine formation and hydrolysis are essential for enantioselectivity for L-amino acids in this process. Gong and Xu[51] tried kinetic resolution of racemic mixtures coupled to ELLE using whole cells of *Bacillus megaterium* ECU1001 of chiral epoxides. It was used an isooctane+aqueous biphasic system to overcome the poor solubility of glycidyl phenyl ether (GPE) in the aqueous phase, resulting in a significant improvement of the enantiomeric ratio from 39.5 to 94.0. In the end, it was possible to obtain (*S*)-GPE with 100% of enantiomeric excess and a yield of 44.5%.

Automatized and continuous operation in countercurrent are easily adapted to ELLE keeping high recoveries and low energy consumption.[48,53] Despite these advantages, the use of large amounts volatile organic solvents makes it not recommendable from an environmental point of view.

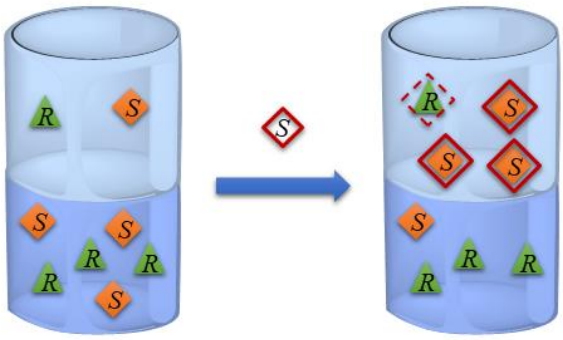

Figure 5 - Schematic representation of enantioseparation with ELLE systems. ▲corresponds to *R* enantiomer, ◆ to S enantiomer and ◊ to a chiral selector specific to *S* enantiomer. Adapted from [32].

1.1.1. Aqueous biphasic systems

In the last 50 years, aqueous biphasic systems (ABS) appeared as an alternative for the traditional ELLE based on volatile organic solvents.[53] This method is considered more attractive due to its high water content. This improves the biocompatibility and environmental impact of the separation and purification processes.[54] The formation of ABS appears from mixing pairs of incompatible and water soluble solutes together in aqueous solution, and above certain concentrations. Knowing the phase diagram (Figure

6), where the biphasic and monophasic regions are respectively located above and below the binodal curve, it is possible to determine the amount of each solute needed to have a biphasic system.

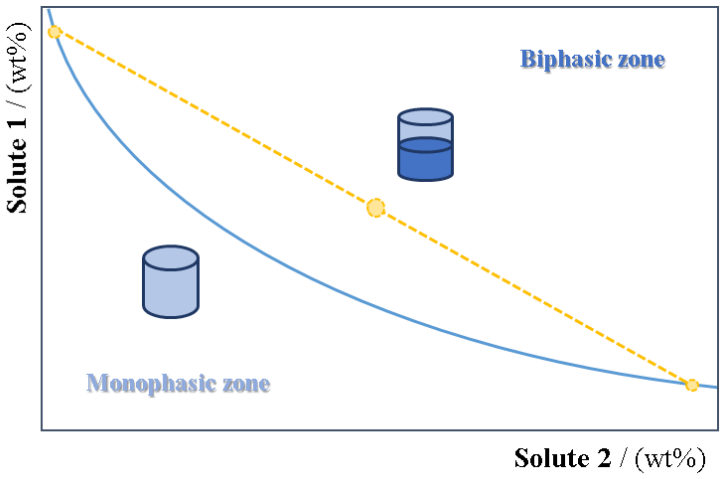

Figure 6 - Representation of a theoretical ABS with the binodal curve represented in blue and the biphasic mixture point in yellow.

The most common combinations are polymer-polymer,[56,57] polymer-salt,[58, 59] or salt-salt,[60,61] but others such as alcohol-salt, [62,63] acetonitrile-carbohydrate, [64,65] polymer-carbohydrate,[65] polymer-amino acid,[66] ionic liquid (IL)-polymer,[68,69] IL-carbohydrate,[70,71] IL-amino acids [72,73], surfactant-amino acids,[73] have appeared recently. Hence, in addition to enhanced biocompatibility, these systems are versatile, because a wide range of phase formers can be used to prepare ABS for any desired application.[75,76] ABS were introduced for chiral resolution purposes by Sellergren and Ekberg in 1985.[76] The authors proposed an ABS formed by two polymers with bovine serum albumin (BSA) as the chiral selector to resolve tryptophan racemates. Nowadays, besides polymer-polymer combinations[76]–[78] others exist, like polymer-salt,[80–82] polar organic solvent-salt,[83,84] polar organic solvent-oligosaccharide,[85,86] and IL-salt.[86]

There are two main modes to apply chiral ABS. One uses a chiral selector which is not essential for the ABS formation [79]–[81,83,84] and the other uses one of the solutes simultaneously as chiral selector and phase former.[78,85,86] In terms of chiral molecules studies, drugs (mandelic acid [81] and its derivatives,[79] non-steroidal anti-inflammatory drugs,[87] antibiotics,[79,89]) and amino acids (tryptophan [83] and

phenylalanine[87,90]) are the most common. A summary of the most used ABS and main chiral selectors used is given in Figure 7.

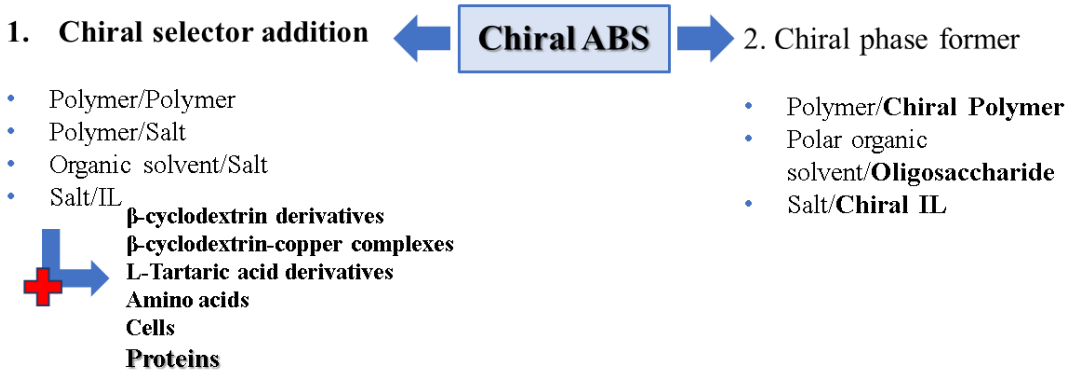

Figure 7- Summary of the types of ABS reported in literature as useful for chiral resolution.

The parameters used to evaluate the enantioselectivity in ABS are the enantioselectivity (α) and the enantiomeric excess (*e.e.*). Enantioselectivity (α) is the ratio between the partition coefficients of each enantiomer (K_S and K_R) indicating that for $\alpha > 1$ an ABS presents enantioselectivity. The enantiomeric excess (*e.e.*) measures the level at which one ABS phase is more enriched in a single enantiomer, meaning that a 50:50 constitution represents an *e.e.* of 0%, while a phase containing a single enantiomer has an *e.e.* of 100%.

The selectivity results obtained up to now with chiral ABS are very promising. The first approach was able to reach *circa* of 58% of *e.e.* for *R*-phenylsuccinic acid when an ethanol+salt ABS with two chiral selectors (L-(+)-tartaric acid diisopropyl ester and hydroxypropyl-β-cyclodextrin) was used. [90] In a surfactant-based system, with Cu_2-β-CD acting as the chiral selector, it was possible to resolve mandelic acid racemates with an *e.e.* of *circa* 68%. [91] This type of micellar systems was the most efficient at resolving mandelic acid racemates in literature. Mandelic acid and its derivatives (α-cyclohexylmandelic) were also resolved using IL+salt ABS + HP-β-CD,[92] polymer+salt ABS + Cu_2-β-CD[79] and alcohol+salt ABS + sulfonated β-CD (with different degrees of substitution).[93] In the work of Ni *et al.*[94], microbial cells are used as chiral selectors in ABS formed by polyethylene glycol with 400 g mol^{-1} of molecular weight (PEG 400) and Na_2HPO_4. (*S*)-(4-Chlorophenyl)-(pyridin-2-yl) methanol, an important intermediate of anti-allergic drug beta histidine, was separated by accumulating substrate/product in the polymer-rich phase. The substrate tolerance and biocompatibility of microbial cells were improved in ABS.

With the second approach, even if simpler technologies are created (no need for an additional chiral selector), the enantioseparation is in general more limited. For instance, chiral polymer+polymer systems obtained a separation factor of 1.27 for mandelic acid. Values 1.5 to 2 fold higher were achieved with organic solvents and oligosaccharides as the chiral phase former for racemic phenylsuccinic acid[85] and zopiclone[84], respectively. Finally, very promising systems were created with combinations of chiral ILs+salts with the addition of copper acetate. Racemic phenylalanine was separated with enantiomeric excesses between 53 – 65%.[87,90] With chiral ILs formed by tropine chiral cation and L-proline chiral anion, the separation was based on the three-point model involving interactions between the anion L-proline, the cation tropine, and D-phenylalanine with copper ion.[89] NMR and DFT measurements were carried to support this hypothesis.

1.2. Proteins as chiral selectors

Proteins are constituted by amino acids, which normally are L-isomers, that could be used as natural chiral selectors in several enantioseparation processes.[95] They are an obvious choice as chiral selectors, because they can discriminate a wide range of chiral drugs (charged or neutral). However, difficulties occur from their high susceptibility to small changes in the medium conditions, mainly of pH, ionic strength and solvents type and amount, inhibiting the enantiomeric recognition. Moreover, it is not easy to describe a simple mechanism for enantiomeric recognition with proteins, because they contain multiple binding sites. All type of interactions, as discussed before, can occur between the target enantiomer and proteins.

Matsunaga[96], for instance, modified the Tryptophan (Trp) 26, the only Trp residue, in chicken 1-acid glycoprotein (1-AGP), with 2-nitrophenylsulfenyl chloride and compared their application for chromatographic columns for the chiral separation of neutral, acidic and basic compounds. Without Trp26, 1-AGP lost its chiral recognition ability for propranolol, alprenolol and oxprenolol and had decreased its enantioselectivity for chlorpheniramine, ketoprofen and benzoin. These results suggest that this protein has a better recognition with Trp26 residue, suggesting that this is responsible for the chiral recognition mechanisms involved. Warfarin binds to the subdomain IIA of Human serum albumin (HSA) and both *R*- and *S*-enantiomers bind in identical conformations and geometric arrangement.[97] In HSA-coumarin complex

formation, coumarin's benzyl-moieties have no role in the HSA chiral recognition. The main difference in coumarin enantiomers is in the acetonyl group conformation and in the different hydrogen bonding occurring between Arginine (Arg) 222 residue and the carbonyl of the coumarin ring (in the *R*-complex) and of the acetonide (in the *S*-complex).[98] The binding to HSA is similar for both enantiomers, which suggests a low degree of enantioselectivity for warfarin enantiomers. Meanwhile, the stereoisomers quinine and quinidine bind differently to BSA as revealed by Liu *et al*.[99] using spectroscopic techniques. BSA has a higher affinity for quinine than for quinidine.[100] The aromatic groups present in these alkaloids as well as the higher proximity to Trp and tyrosine (Tyr) residues of quinine were shown to dictate the enantiomeric recognition.[100,101]

These enantiomeric recognition mechanisms are useful for the development of successful enantioseparation techniques. BSA and HSA are the most commonly used proteins as chiral selectors. The most common techniques using proteins as chiral selectors are HPLC and capillary electrophoresis (CE), as shown in Table 1. However, other techniques were studied, using hemoglobin in electrochemiluminescence [101], or even antibodies in bionanotube membranes.[102]

Table 1 - Some examples of proteins as chiral selectors used in different enantioseparation methods.

Protein	Enantiomers	Enantioseparation method	Ref.
BSA	Propanol, ofloxacin, verapamil	CE	[103]
	Tryptophan derivates	CE	[104]
	Tryptophan	Membranes	[105]
	Propanol, Tryptophan	CE	[106]
	Profen	HPLC	[107]
	Penicillamine	Electrochemical methods	[108]
	Tryptophan	Counter-current chromatography	[109]
	Tryptophan, Hydrobenzoin, Phenylalanine, Mandelic acid	HPLC	[110]
	Propanolol	Electrochemical sensing layer	[111]
	Ibuprofen, ofloxacin	HPLC	[112]
	Tryptophan	Ultrafiltration/Membrane	[113]
HSA	D,L-dansyl aminoacids	HPLC	[114]
	Cetirizine	HPLC	[115]
	Tryptophan	Affinity ultrafiltration	[116]
	Propanol, ofloxacin, verapamil	CE	[103]
AGP	R,S-propanol	HPLC	[117]
Trypsin	Amino acids	HPLC	[118]
Pepsin	Trimipramine, propranolol, promethazine, verapamil, cloperastine, oxprenolol, pindolol	Capillary electrophoresis	[119]
Casein	DNP-amino acids	Capillary electrophoresis	[120]
Lysozyme	Tryptophan, dansyl-leucine, PTH-aspartatic acid	Tubular capillary electrochromatography	[121]
Hemoglobin	Penicillamine	Electrochemioluminescence	[101]
Antibody	4-[3-(4-fluorophenyl)-2-hydroxy-1-[1,2,4]triazol-1-yl-propyl]-benzonitrile	Bionanotubes membranes	[102]
Ovoglycoprotein	Eparipsome, tolperisome, chlorpheniramine, verapamil	CE	[122]

1.2.1. Aqueous biphasic systems containing proteins as chiral selectors

Proteins have been employed in chiral ABS for enantioseparation since 1985.[123] With the advances on ABS application for protein separation, it is nowadays known that these systems can maintain the protein structure, stability and activity if well designed.[55,125] It is also known that proteins interact with the surrounding molecules present in an ABS phase, mainly by hydrogen bonding, electrostatic interactions, π-π stacking and hydrophobic interactions. Thus, salting-out and the hydrophobicity are the main phenomena justifying the protein extraction in ABS. By judicious choice of the phase formers it is possible to concentrate the protein into a single phase.[74] The maintenance of proteins' structure, stability and activity and their concentration into one phase make ABS containing proteins as good candidates for enantioseparation processes.

Arai *et al.* [78] used BSA and ovomucoid as chiral discriminator in an ABS formed by dextran 40 and PEG. Ofloxacin enantiomer was recognized by BSA with an *e.e.* of 62%, but the enantiomers of carvedilol (b-blocker) and DG-5128 (a new oral hypoglycemic drug) were poorly recognized by ovomucoid. It was possible to obtain a *R*-ofloxacin enriched sample by purification by thin-layer chromatography. In the work of Shinomiya *et al.*[80], BSA was used as chiral discriminator to separate enantiomers of kynurenine in an ABS constituted by PEG 8000 and Na_2HPO_4. BSA was concentrated in the polymer-rich phase. The separation was done with a high-speed countercurrent chromatography, where the lower Na_2HPO_4-rich mobile phase eluted the L- kynurenine prior to the D-isomer, with a peak resolution of 0.94.[123] This demonstrated the affinity of BSA to the D-isomer, since the L-isomer was eluted in the salt phase and the BSA would be present in the polymer-rich phase. The works presented in literature are in general promising and report high *e.e.* or α values when compared to works involving other chiral selectors.

Racemic ofloxacin was also enantioseparated with L-TA + HP-β-CD in a polymer+salt ABS with α = 1.32.[88] D,L-tryptophan was enantioseparated with β-CD in an ethanol+salt ABS with α = 1.38.[83] As presented in Table 2, the enantioselectivity of L-tryptophan with BSA using a polymer+polymer ABS is better, with an enantioselectivity of α = 3.1. Thus, proteins can be efficient chiral selectors in ABS.

Table 2 - Proteins as chiral selector in chiral ABS.

Protein	**ABS**	**Enantiomers**	**Enantioselectivity**	**Ref.**
Ovomucoid	PEG+Dextran	Carvedilol	*Not occurred*	[78]
	PEG+Dextran	DG-5128	*Not occurred*	[78]
BSA	PEG+Na_2HPO_4	D,L-Kynurenine	*Not determined*	[80]
	PEG+Dextran	Ofloxacin	*e.e.* = 62%	[78]
	PEG+Dextran	L-tryptophan	α = 3.1	[123]

In spite of the promising results achieved, few articles exists and the role of proteins as chiral selectors in ABS remains misunderstood. Moreover, having in mind the multiple proteins that are known up to date, the works reported in literature are limited, with BSA being the most studied. However, screening the proteins to find the ideal one to be used as chiral selector in ABS for a specific resolution application will be only done by performing multiple experiments, taking a long time and spending large amounts of reagents. In order to overcome these problems, there is an urgent demand to find computational tools able to predict protein-enantiomers interactions.

1.2.2. Molecular docking - virtual screening of proteins as chiral selectors

Molecular Docking (MD) is a virtual screening method that has been receiving a crescent attention mainly because it is believed that this method allows understanding the interactions between small chemical molecules and a protein at molecular level, in a fast, easy and highly accurate way (Figure 8).[125] MD algorithms provide quantitative predictions of docking binding energy, allowing predicting the most stable binding affinity between several molecules and proteins. MD is also applied to explain fundamental biochemical processes and their related experimental data, since it allows predicting how ligand-protein complexes occur.[126]

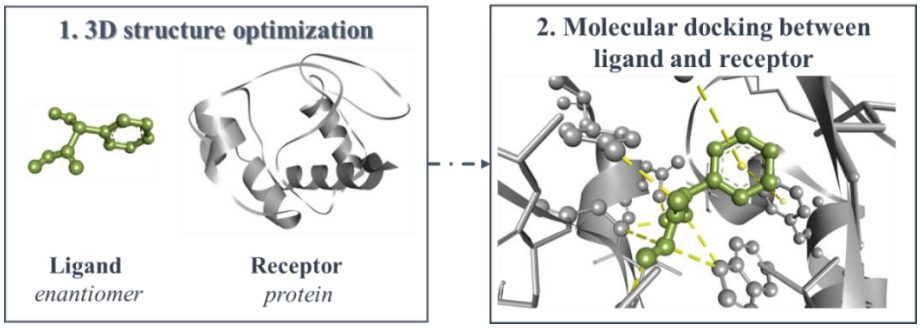

Figure 8- Scheme of the molecular docking method: ligand (green); receptor (grey); molecular interactions (yellow dashed line).

MD has demonstrated to be a promising approach to identify protein enantioselectivity, by predicting enantiomer-protein (ligand- receptor) affinities, for example between acetochlor-interleukin 1,[127] diastereomeric heterodimers-acetylcholinesterase,[128] 4-phenyl-2-dimethylaminotetralin-G protein[129] and omeprazole-cytochrome P450.[130] In Table 3, several MD algorithms employed to explain protein enantiorecognition mechanisms are displayed. Each method applies different algorithms.[132–134] In some of these algorithms the ligand is incrementally designed, starting from docked ‘base fragment’, such as in DOCK[134] and FlexX.[135] However, using AutoDock[136] and Genetic Optimization for Ligand Docking (GOLD),[137] the interactions between the ligand and full receptor are taken into account. AutoDock works with the Lamarckian genetic algorithm (GA),[138] the SLIDE and Glide are supported by descriptor matching, FRED[139] (Algorithm that apply shape matching using gaussian functions) and Surflex Dock that is based on surface-based molecular similarity.[140] Besides ligand flexibility, receptor torsions are desirable in order to allow conformational changes that are required to bind the ligand with lower bind energy (induced fit phenomenon).[141] Due to the computational complexity, the receptor flexibility is unusual in most of the docking methods. FlexE[142] works using multiple receptor conformations, allowing any atoms torsion in a defined buffer region between the fixed and mobile regions. Another parameter is the software ability to support different number of atoms and rotatable bonds in the ligand structure. In summary, there are programs that maximize shape complementarity, often based on geometric criteria, and others incorporating an energy-driven or stochastic algorithm. Auto Dock presents a higher performance ability compared to other softwares, for example Glide,[131] that is limited at 200 atoms on ligand and the maximal number of rotatable bond of 35. In Auto Dock, the receptors are rigid

structures and the ligands are treated as flexible molecules with the number of active rotatable bonds ranging from 0 to 32. Moreover, there is no number of atoms limit, being this dictated by the receptor grid box.

AutoDock Vina is a MD software, an update of AutoDock4, aiming to improve the binding mode accuracy.[143] AutoDock Vina predicts binding affinities between ligand and receptor, that can be used to predict where and which interactions occur at the level of receptor surface and which conformation could be the best fitting. Further speed-up is achieved from parallelism, by using multithreading on multicore machines. AutoDock Vina was already applied to select inhibitors with favorable interactions with 2-Transenoylacyl carrier protein reductase in *Mycobacterium tuberculosis*.[144] The docking predicted structure ligand-receptor helped to identify a group of natural potential inhibitors which displayed high binding affinities for protein. Thus, these molecules could potentially inhibit the protein and be the basis to develop, for example, new anti-tuberculosis drugs. AutoDock Vina was more accurate and ten times faster compared to last version AutoDock4. [145] Based on this, AutoDock Vina displayed more advantages compared to traditional MD softwares, promoting accurate predictions with no cost of acquisition.

Since this type of software can predict the affinities and interactions established between the enantiomers and proteins, it can be useful to predict the ability of a protein to act as chiral selector in ABS.

Table 3- Some examples of ligand-protein complexes prediction using Molecular Docking methods.

Method	**Protein**	**Enantiomers**	**Application**	**Ref.**
Molegro Virtual Docker	Human serum paraoxonase 1	Sarin, soman and VX	Biocatalysis	[146]
Maestro	Potato epoxide hydrolase	Benzyloxirane and 3-phenylpropane-1,2-diol	Biocatalysis	[147]
	hCE2 and mfCES2v3	*O*-*n*-valeryl propranolol, *O*-2-methyl-*n*-butyryl propranolol and *O*-3-methyl-*n*-butyryl propranolol	Biocatalysis	[148]
Molecular Operating Environment	Cereblon	Thalidomide, lenalidomide, and pomalidomide	Drug design	[149]
	*Ldis*PBP1 and *Ldis*PBP2	(7,8)-epoxy-2-methyloctadecane, Dis), 5-oxadisparlure (5-oxaDis), 10-oxadisparlure (10-oxaDis), 5-thiadisparlure (5-thiaDis) and 10-thiadisparlure(10-thiaDis)	Binding assays	[150]
AutoDock 4.0	Cucumber mosaic virus coat protein (CMV-CP)	α-aminophosphonates	Antiviral activity	[151]
AutoDock Vina	Penicillin G acylase	N-phenylacetyl amino acid	Biocatalysis	[152]
Extra Precision (XP) Glide 4.5	Cathepsin L –like cysteine proteases	Thiocarbazate	Binding assays	[153]
QUANTUM 3.3	*Leishmania donovani* PTR1 (LdPTR1)	Monastrol	Drug Design	[154]

1.3. Carbohydrates as chiral selectors

Another example of chiral selectors are carbohydrates. They present chirality being promising alternatives for chiral resolution applications. Cyclodextrins (CD) are the most common carbohydrates derivatives used as chiral selector in techniques such as capillary electrophoresis (CE) and counter current chromatography (CCC). Maruszak *et al.* [155] have incorporated β-CD in CE, showing that this was capable of discriminating different neurotransmissors as DOPA or phenylalanine. Polysaccharides and their derivates are extensively used as chromatographic chiral selectors in chiral stationary phases for the separation of enantiomers by HPLC. In the work of Matarasvihili *et al.* [156], the separation of 10 chiral arylpropionic acids derivates (AADs) using polysaccharide-based chiral HPLC columns was successfully achieved. Cellulose and amylose were studied, being the only difference in the linkage between the glucopyranose units (α in amylose and β in cellulose). This difference affected the chiral recognition, changing the retention time of the enantiomers being separated. In the work of Sun and Yuan [157], arabinose and ribose were integrated in different silica-based analytical columns. In the case of arabinose, benzoin enantiomers were resolved and in the ribose column there was a discrimination of trans-stilbene. This suggests that different carbohydrates can discriminate different compounds.

These enantiomeric recognition mechanisms are utile for the development of successful enantioseparation techniques. Sucrose and cyclodextrins and their derivates are the most commonly used carbohydrates in enantioseparation processes. The most common technique with carbohydrates as chiral is CE, as shown in Table 4.

Table 4 - Some examples of carbohydrates as chiral selectors used in different enantioseparation methods.

Type	Carbohydrate	Enantiomers	Enantioseparation method	Ref.
Monosaccharide	Glucose	Binaphthyl enantiomers (BNA, BNP)	CE	[158]
		D-serine	CE	[159]
	Mannose	Binaphthyl enantiomers (BNA,BNP)	CE	[158]
	Fructose	D-serine	CE	[159]
	Galactose	Binaphthyl enantiomers (BNA, BNP)	CE	[158]
	Ribose	Trans-stilbene	HPLC	[157]
	Xylose	Binaphthyl enantiomers (BNA, BNP)	CE	[158]
	Arabinose	Binaphthyl enantiomers (BNA, BNP)	CE	[158]
		Benzoin	HPLC	[157]
Disaccharide	Sucrose	Metalaxyl enantiomers	UV-vis spectroscopy	[160]
		Phenylalanine	UV-vis spectroscopy	[161]
		D-serine	CE	[159]
	Maltose	Citalaprom (CIT)	CE	[162]
	Lactose	D-serine	CE	[159]
Polysaccharide	Glycogen	Basic drugs (CIT, NEF, CET), acid drugs (IBU)	CE	[163]
	Cellulose	Naproxen, ibuprofen, fenoprofen, indoprofen	HPLC	[156]
	Amylose	1,1'-binaphthyl-2,2'-diyl hydrogen phosphate (BDHP)	CE	[164]
		Naproxen, ibuprofen, fenoprofen, indoprofen	HPLC	[156]
Derivate	CD	Serotonin, phenylalanine, dopamine, adrenaline, ephedrine, propranolol and DOPA	CE	[155]
		(R,S)-naproxen	CCC	[165]
		Mandelic acid derivates enantiomers	HPLC	[166]
		Phenylsuccinic acid	CCC	[167]

1.3.1. Aqueous biphasic systems with sugar as phase former and chiral selector

Carbohydrates are compounds easily found in nature that present no charge, are biodegradability and lack toxicity, what makes their application in ABS an eco-friendly alternative to the use of the common high charge density salts. Carbohydrates have high affinity for water and salting-out aptitude, due to the several –OH groups with donor/acceptor character that they possess in their structures. Polymers [65] and ILs [69] are examples of phase formers with which carbohydrates can be combined to form ABS. Sadeghi *et al.* [65] screened several carbohydrates with PPGs and PEGs of multiple molecular weights to develop novel, more biocompatible ABS. Also, Taha *et al.* [168] applied polymer+sucrose-based ABS to extract BSA, with enhanced biocompatibilities achieved. To overcome the high viscosity of the polymer-rich phase, Zhang et al. [169] showed the possibility of creating 1-Butyl-3-methylimidazolium tetrafluoroborate ([C_4mim][BF_4])+fructose-based systems. Since [C_4mim][BF_4] is hydrolyzed in aqueous medium, Freire et al. [69] tested different carbohydrates paired with the water-stable 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([C_4mim][CF_3SO_3]) to form ABS. From these works, and others where polar organic solvents were used [62,63], the possibility of pairing carbohydrates with multiple other phase forming agents creates the possibility to have a versatile separation platform.

The works reported in literature for the application of sugar-based ABS in chiral resolution lay mainly on their use as chiral selectors added as an extra compound [81,87]. These are overviewed in Table 5. CD and their derivatives are the only used compounds, with promising results of enantioselectivity up to 68% of enantiomeric excess. Some works [76,83,84] report the use of newly synthesized CD derivatives simultaneously as phase formers and chiral selectors, however with lower enantioselectivities (up to 33% of enantiomeric excess). The main advantage of this second option, as already mentioned, is the simpler constitution of the ABS. However, the CD derivatives used so far involve complex synthetic routes [154,164,165] and the possibility of using less complex, naturally occurring carbohydrates as phase formers and chiral selectors for chiral resolution in ABS was never reported.

Table 5 - Carbohydrates as chiral selectors in chiral ABS.

Carbohydrate	**ABS**	**Enantiomers**	**Enantioselectivity**	**Ref.**
β-CD	PEG2000+$(NH_4)_2SO_4$	Mandelic acid; Phenylalanine	*α=2.46; α=1.53 and e.e.= 12.8%*	[81], [170]
	Ethanol+$(NH_4)_2SO_4$	Tryptophan	*α=1.38*	[83]
Cu_2-β-CD complex	Triton X-114	Mandelic acid	*e.e.=68%*	[91]
	PEG 6000+ $Na_3C_6H_5O_7$	α-cyclohexylmandelic acid	*α=1.36*	[79]
Sulfonated-β-CD	Ethanol+$(NH_4)_2SO_4$	Mandelic acid	*α=1.69; e.e.=16.3%*	[93]
hydroxypropyl-β-CD	$[C_4mim][BF_4]$+ $(NH_4)_2SO_4$	α-cyclohexylmandelic acid	*α=1.59*	[92]
	PEG2000+$(NH_4)_2SO_4$	Phenylsuccinic acid; ofloxacin	*α=1.99 and e.e.=23.5%; α=1.32 (with second chiral selector)*	[88], [171]
	Ethanol+$(NH_4)_2SO_4$	Phenylsuccinic acid	*α=1.42; α=4.06 and e.e.=57.9% (with second chiral selector)*	[82], [90]
Thermosensitive polymer Poly(MAH-β-CD-co-NIPAAm)	Poly(MAH-β-CD-co-NIPAAm)+DexT40	Mandelic acid	*α=1.27*	[77]
carboxymethyl-β-CD	1-propanol+ carboxymethyl-β-cyclodextrin	Zopiclone	*α=2.58 and e.e.=32.7%*	[84]
Sulfobutyl ether-β-CD	Acetone+ Sulfobutyl ether-β-CD	Phenylsuccinic acid	*α=2.1 and e.e.=31.7%*	[85]

1.4. Scopes and objectives

Given the good ability of ABS to keep proteins structure and stability if well optimized,[56] the use of proteins in ABS for chiral resolution approaches can be seen as a promising alternative. Although some works have already reported this approach, the type of ABS tested is still limited if considering the structural diversity that they possess. Systems formed by polymer-polymer[56,172] and polymer-salt [173], polymer-IL [174] or even salt-IL [175] are some of the combinations already used to purify proteins. However, the range of proteins studied is limited (only BSA and ovomucoid are reported) as well as and the understanding on their mechanisms to act as chiral selectors within these systems.[77,79] Under this scenario, the main objective of this thesis is to enlarge the number and type of ABS and proteins that can be used for chiral resolution. In order to accomplish this objective, several tasks were developed according to Figure 9.

First, ABS using proteins as chiral selectors will be focused. A bioinformatic tool will be used to predict the interactions between the MA enantiomers and different proteins, trying to find the ideal protein to enantioseparate MA. AutoDock Vina will be the autodocking tool used, since it is able to distinguish the interactions that occur between a certain protein and the two enantiomers of a certain compound in an accurate, quick and free way. Based on such predictions, two different proteins, one ideal, protein that display enantioselectivity, and other not ideal, will be chosen to screen different types of ABS (polymer-polymer, polymer-salt, IL-salt, IL-polymer, among others) for chiral resolution purposes.

Owing to the several pairs of phase formers that can be used to form ABS, it is possible to identify a poor exploitation of these systems for enantioseparations. While the most conventional polymer-polymer and polymer-salt pairs are well-studied, others remain poorly studied. Also, some naturally occurring chiral compounds can be used to form ABS, where carbohydrates [69] and amino acids [71,174] are included. Moreover, the operational simplicity of the previous approach (where proteins are used as an extra chiral selector) can be improved. In this ambit, carbohydrates can be applied as natural phase formers and chiral selectors in ABS. Particularly, systems composed of sucrose and polymers or ILs will be exploited.

In order to develop this enantioseparation platform, 2-hydroxy-2-phenylacetic acid, commonly known as mandelic acid (MA), was used as the model chiral compound. MA

is an aromatic compound with the molecular formula $C_8H_8O_3$ (Figure 1), with the *R* and *S* enantiomers differing in the C2, with a rotation on this position. It was chosen as the model drug for this study, since it is widely used by several industries and their two enantiomers do not display the same activity what justifies the need for their separation. The enantiomer *R* is used in pharmaceutics as intermediate for the synthesis of antibiotics like penicillin[177] and in cosmetics for skin care products, to treat cases of photo-aging and acne due to its antibacterial action.[178] *R*-MA is also used as chiral resolving agent for the synthesis of anti-tumour agents.[179]

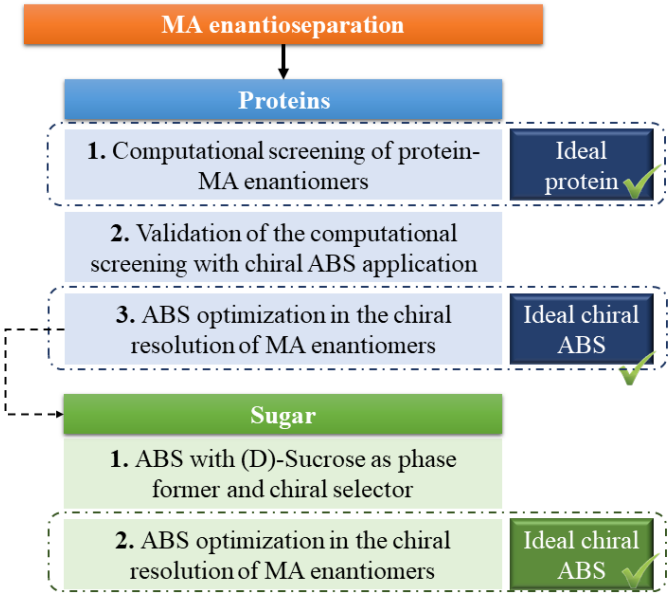

Figure 9 - Summary of the tasks and objectives involved in the present thesis.

2. Experimental section

2.1. Chemicals and biomolecules

The *R* and *S* enantiomers of MA (purity = 99 wt%) were both acquired from Acros Organics. The Cyt C from equine heart (purity > 95 wt%) was obtained from Sigma-Aldrich. The BSA (fatty acid free) was purchased from Fisher Scientific. A phosphate buffer saline (PBS) solution was prepared at pH 7.4, using PBS tablet from Sigma-Aldrich.

The polymers used in ABS preparation were polyethylene glycol M_n 400 and 3350, PEG 400 and PEG 3350, polypropylene glycol M_n 400, PPG 400, and dextran from *Leuconostoc spp*. M_n 450 000-650 000, Dex 500. All of them were acquired from Sigma- Aldrich. The salts used were sodium sulphate, Na_2SO_4 (purity = 99 wt%), from Sigma-Aldrich and tri-potassium citrate monohydrate, $K_3C_6H_5O_7$, from Prolabo. Citric acid, $C_6H_80_7$ (purity = 100 wt%) was purchased at Fisher Scientific. The two latter compounds were used together to prepare citrate buffer at pH 7. The imidazolium-based ILs employed were acquired at IoLiTec and were 1-butyl-3-methylimidazolium chloride, [C_4mim]Cl (purity = 99 wt%) and 1-butyl-3-methylimidazolium trifluoromethanesulfonate, [C_4mim][CF_3SO_3] (purity = 99 wt%). The cholinium-based compounds, cholinium dihydrogen citrate, [Ch][DHCit] (purity 99 wt%)and cholinium acetate, [Ch][Ac] (purity > 99 wt%) were from Sigma-Aldrich, Acros Organics and IoLiTec, respectively. The remaining ILs used belong to the phosphonium and quaternary ammonium families, namely triisobutyl(methyl)phosphonium tosylate, [$P_{i(444)l}$][Tos] (purity > 95 wt%), tetrabutylphosphonium chloride, [P_{4444}]Cl (purity > 95 wt%), both from Cytec and tetrabutylammonium chloride, [N_{4444}]Cl (purity ≥ 97 wt%) from Sigma-Aldrich. All the chemical structures of polymers, ILs and (D)-sucrose are presented in Figure 10.

The mobile phase used in HPLC analysis was composed of L-phenylalanine, L-Phe (purity = 99 wt%) acquired from Alfa Aesar, copper sulfate pentahydrate, $CuSO_4{\cdot}5H_2O$ (purity = 99.5wt%) purchased from BDH chemicals, methanol (HPLC grade) was from CHEM-LAB and ultra-pure water, which was double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

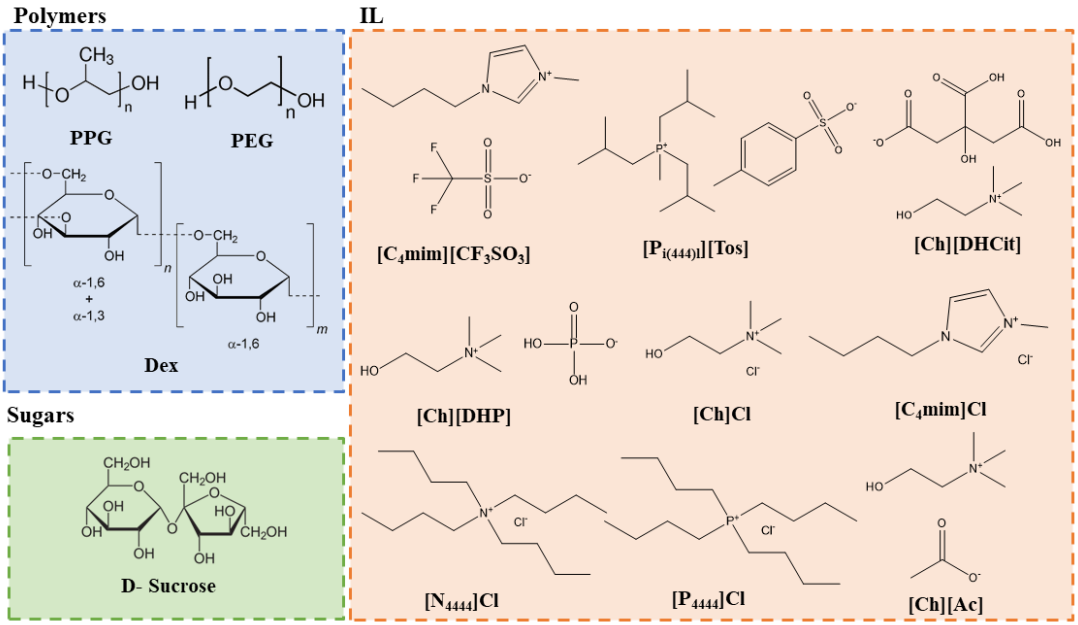

Figure 10 - Chemical structures of the compounds used in the ABS preparation.

2.2. Experimental

2.2.1. Partition studies of mandelic acid enantiomers in different chiral ABS

Different two-phase mixture compositions were used in the preparation of ABS, according to the position of the binodal curves reported in literature (Table 6 to 8). Due to the distinct aptitude of different systems to form two phases, distinct mixture points were selected to perform this initial screening. The total mass of each system was 3 grams and the correct amount of each phase former was added within an uncertainty of 10^{-4} g, according to Table 6 and 7. Two studies were done, one where the partition of the protein and the enantiomers was performed separately and another where the protein and the enantiomers coexisted inside the same ABS. The solution containing the protein and/or the enantiomers was prepared at a concentration of 1 g.L $^{-1}$ of protein (Cyt C or BSA) and/or 10.4 g.L^{-1} of each mandelic acid enantiomer. At the end, *R*-MA and *S*-MA are present at a concentration of 4.2 mg *per* g of ABS. These components were prepared in PBS. The solution composed by enantiomers and protein was left overnight, to promote interactions between them and the thermodynamic equilibrium for the complete migration of each enantiomer for each phase. The two phases (top and bottom) were separated and weighed for posterior quantification of enantiomers in HPLC. In Tables 6 to 8 are represented the different ABS with the composition and phases identification.

The parameters calculated to evaluate the ability of the different proteins to separate the two enantiomers of MA are indicated in Equations 1-3 as follows.

$$E.E._{R-MA}\% = \frac{m_{R-MA_{CS\,rich-phase}}}{m_{0_{R-MA}}} \times 100 \quad (1)$$

$$E.E._{S-MA}\% = \frac{m_{S-MA_{CS\,rich-phase}}}{m_{0_{S-MA}}} \times 100 \quad (2)$$

$$e.e.\% = \frac{(m_{R-MA\,CS\,rich-phase} - m_{S-MA\,CS\,rich-phase})}{(m_{R-MA\,CS\,rich-phase} + m_{S-MA\,CS\,rich-phase})} \quad (3)$$

In these equations, $m_{\text{R-MA CS rich-phase}}$ and $m_{\text{S-MA CS rich-phase}}$ are, respectively the mass of the *R* and *S* enantiomers in the chiral selector rich-phase. The $m_{0\text{R-MA}}$ and $m_{0\text{S-MA}}$ corresponds to the initial mass of *R* and *S* enantiomers present in the ABS. $m_{\text{R-MA CS-rich - phase, and}}$ $m_{\text{S-MA CSrich-phase}}$ are, respectively, the mass of *S* and *R* enantiomers in chiral selector rich-phases. The chiral selector rich-phase corresponds to the phase of the ABS wherein the protein/(D)-sucrose tend to be more present, in other words, the most appropriate "phase" to attest the enantioselective patterns.

Table 6 - Mixture points composition used in the partition studies of MA enantiomers using ABS containing cytochrome C as the chiral selector.

ABS	**Phase formers**		**Mixture point composition**				**Phases identification**		
Type	**1**	**2**	**1 / (wt%)**	**2 / (wt%)**	***R/S*-MA+Cyt C aqueous solution / (wt%)**	**PBS / (wt%)**	**Top phase**	**Bottom phase**	**Ref.**
Polymer-salt	PEG400	Na_2SO_4	22	12	40	26	PEG	Salt	[180]
Polymer-IL	PPG400	[Ch][DHP]	30	30	40	-	PPG	IL	[181]
Polymer-polymer		PEG400	25	35	40	-	PPG	PEG	[56]
	PEG3350	Dex500	5	15	40	40	PEG	Dex	[172]
IL-salt	[C_4mim]Cl	$C_6H_5K_3O_7$	22	33	40	5	IL	Salt	[182]

Table 7 - Mixture points composition used in the partitioning studies of mandelic acid enantiomers using ABS containing BSA as the chiral selector.

ABS	**Phase formers**		**Mixture point composition**				**Phases identification**		
Type	**1**	**2**	**1 / (wt%)**	**2 / (wt%)**	***R/S*-MA+BSA aqueous solution / (wt%)**	**PBS / (wt%)**	**Top phase**	**Bottom phase**	**Ref.**
Polymer-IL	PPG400	[Ch][Ac]	30	30	40	-	PPG	IL	[181]
		[Ch][DHCit]	30	30	40	-	PPG	IL	
Polymer-salt		Na_2SO_4	32	12	40	16	PPG	Salt	[183]
Polymer-sugar		(D)-Sucrose	30	30	40	-	PPG	(D)-Sucrose	[168]
Polymer-polymer		PEG400	35	25	40	-	PPG	PEG	[56]
	PEG3350	Dex500	5	15	40	40	PEG	Dex	[172]
Salt-IL	$K_3C_6H_5O_7$/ $C_6H_5O_7$ at pH 6	[$P_{i(444)1}$][Tos]	15	30	40	15	IL	Salt	[175]
		[P_{4444}]Cl	15	30	40	15	IL	Salt	
		[N_{4444}]Cl	15	30	40	15	IL	Salt	

Table 8- Mixture points composition used in the partition studies of MA enantiomers using ABS composed of sucrose.

	Phase formers		**Mixture point composition**				**Phases identification**		
Type	**1**	**2**	**1 / (wt%)**	**2 / (wt%)**	***R*/*S*-MA aqueous solution / (wt%)**	**PBS / (wt%)**	**Top phase**	**Bottom phase**	**Ref.**
Polymer-sugar	PPG400	(D-)Sucrose	20	40	40	-	PPG	(D)-Sucrose	[168]
			30	30	40	-			
			26	26	40	8			
IL-sugar	$[C_4mim][CF_3SO_3]$		20	40	40	-	IL		[69]

2.2.2. Mandelic acid enantiomers quantification

The quantification of the two enantiomers of MA was done by HPLC-DAD by a method developed and validated in our research group, based on a protocol previously reported in literature.[184] The liquid chromatograph was a HPLC Elite LaChrom (VWR Hitachi) possessing a diode array detector (DAD) l-2455, column oven l-2300, auto-sampler l-2200 and pump l-2130. The analytical column was constituted by a sorbent LiChrospher 100 RP-18 (5μm) and cartridge LiChroCART 250-4 HPLC-Cartridge, linked to a 5 μm, 4 mm × 4 mm guard column with the same stationary phase. The analytical column was from Merck. The mobile phase was chiral and it was prepared by mixing 15 wt% of methanol and 85 wt% of ultrapure water, containing 2 mM of L-phenylalanine and 1 mM of $CuSO_4$. Whenever necessary, the pH of the mobile phase was corrected to 4.00 by adding an aqueous solution of ammonia (5 wt%). The mobile phase was then filtered under vacuum using regenerated cellulose membrane filters (0.45 μm) and degassed in an ultrasound bath. The chromatographic separation was done under isocratic mode, at a flow-rate of 0.8 $mL.min^{-1}$ for 20 minutes. The injection volume was 20 μL, the DAD detector measured at 270 nm, the column oven temperature was 22 ºC and the autosampler temperature was 25 °C. The quantification was based on a calibration curve previously determined (Appendix A), based on 8 standard solutions of known concentrations of each enantiomer (10 – 1000 $mg.L^{-1}$) in water:methanol (85 wt%:15 wt%) against the corresponding peak areas. Each system was done in quadriplicate and at least two injections *per* sample were done.

All ABS phases were filtrated and diluted 100 times in a solution of water (85 wt%) and methanol (15 wt%). By integrating the peaks, it was possible to quantify each enantiomer, once they possess different retention times. *R*-MA elutes first at 11 min followed by *S*-MA which elutes at 13 min.

2.2.3. Proteins' quantification

For proteins' quantification, the same mixtures points were prepared as those indicated in Tables 6 and 7 with the presence of the protein solution (1 $g.L^{-1}$) in PBS only. The phases were separated and weighed. Cyt C and BSA were quantified at 410 nm and 280 nm, respectively, using a UV-Vis microplate reader (Synergy HT micro-

plate reader - BioTek). The extraction efficiency of the proteins was calculated through Equation 4. Each sample was analysed at least three times.

$$E.E._{prot}\% = \frac{Abs_{prot-rich} \times w_{prot-rich}}{(Abs_{prot-rich} \times w_{prot-rich}) + (Abs_{other} \times w_{other})} \times 100 \qquad (4)$$

$Abs_{prot\text{-}rich}$ and Abs_{other} are the values of absorbance in the protein rich phase and the other phase, top or bottom, depending on the protein migration. Blank controls (ABS where no protein was added) were applied to discount any interference coming from the phase constituents. $w_{prot\text{-}rich}$ and w_{other} is the weight of the protein rich-phase and the other phase, respectively. the extraction efficiency of the Cyt C was evaluated for five systems: PEG400+Na_2SO_4, PPG400+[Ch][DHP], PEG400+PPG400, PEG3350+Dex500 and [C_4mim]Cl+$C_6H_5K_3O_7$. For BSA, it was only calculated $E.E._{prot}$ for PEG3350+Dex500, PEG400+PPG400 and PPG400+Na_2SO_4.

2.2.4. Molecular docking

The interaction sites of proteins with the MA enantiomers were identified using the Auto-dock vina 1.1.2 program.[143] The crystal structures of BSA (4F5S), CytC (1HRC), Human serum albumin (HSA) (1E7I), Bromelain (1W0Q), Hexokinase II (1IG8), Amylase (1UA7), Green fluorescent protein (GFP) (4KW4), Laccase (1KYA), Lysozyme (4YM8), Ovalbumin (1OVA) and Phycoerythrin (1EYX) were used in the molecular docking, where the Auto DockTools (ADT)[138] was used to prepare the proteins input files by merging non-polar hydrogen atoms, adding partial charges and atom types. Ligand (*S*- and *R*- mandelic acid) 3D atomic coordinates were computed by Gaussian 03w and ligand rigid root was generated using ADT, setting all possible rotatable bonds defined as active by torsions. The grid center at the center of mass (x-, y-, and z-axes, respectively) to cover the whole interaction surface of: BSA (126 Å × 68 Å × 96 Å), CytC (40 Å × 40 Å × 40 Å), HSA (92 Å × 52 Å × 92 Å), Bromelain (58 Å × 46 Å × 48 Å), Hexokinase II (62 Å × 80 Å × 56 Å), Amylase (52 Å × 54 Å × 76 Å), GFP (48 Å × 60 Å × 44 Å), Laccase (54 Å × 64 Å × 72 Å), Lysozyme (40 Å × 46 Å × 46 Å), Ovalbumin (74 Å × 46 Å × 60 Å) and Phycoerythrin (50 Å × 64 Å × 40 Å). The binding model that has the lowest binding free energy was searched out from 9 different conformers for each ligand (MA enantiomers).

3. Results and discussion

3.1. Protein as chiral selectors in ABS for MA enantioseparation

The molecular docking was already used in other works to predict interactions and affinities between proteins and chiral ligands, as overviewed in Table 3. A screening of several proteins using molecular docking was done to predict the interactions with MA enantiomers. The proteins incorporated as chiral selectors in the ABS were chosen according to the results obtained by molecular docking, namely BSA and Cyt C.

The ability of ABS for the extraction and purification of BSA and Cyt C was already shown[68] by using polymer-salt,[185], IL-salt,[186,174], polymer-polymer[187][54] and polymer-IL[168] systems. In this context, and considering the stability of the proteins in these systems, a screening of several types of ABS composed of polymer-polymer, polymer-IL, salt-IL, polymer-sugar and containing Cyt C or BSA as chiral selectors was performed. This allowed evaluating the ABS chiral resolution ability for racemic MA regarding two main aspects: (i) the interactions occurring between the MA enantiomers and the protein and (ii) the type of phase formers yielding the adequate interactions (electrostatic, hydrophobic, hydrogen bonding) for chiral resolution to take place. At the end, it was also possible to conclude on the molecular docking ability to be used as a predicting tool to design chiral resolution approaches based on ABS containing proteins as the chiral selectors.

3.1.1. Molecular docking

In order to evaluate the enantiomers interactions with proteins, molecular docking using Autodock Vina was applied. The 3D structures were prepared using Discovery Studios 2016. Several proteins were tested, namely Cyt C, BSA, HSA, Bromelain, Hexokinase II, Amylase, Green Fluorescent Protein (GFP), Laccase, Lysozyme, Ovalbumin and Phycoerythrin. All proteins' 3D structure was obtained from RSCB Protein Data Bank (PDB). The objective was to identify the protein capable to establish a selective interaction with one of the MA enantiomers. Therefore, different parameters were observed to select the ideal protein to enantioseparate MA such as, the interaction of amino acids residues, docking affinity ($kcal.mol^{-1}$), type of interactions and geometry distance (Å) of each MA enantiomers. The results are presented in Tables B.1-B.9 and Figures B.1-B.9 in Appendix B. Since the lowest absolute value of affinity bind pose ($kcal.mol^{-1}$) for MA enantiomers was similar for both structures (*R*- and *S*- MA), the

specific interactions and interacting amino acids residues provide more details about the docking affinity on protein structure. Based on this, the proteins were classified into two categories: competitive and non-competitive (similar and non-similar amino acid residues interaction, respectively). The proteins showing MA-enantiomers competitive behaviour were the bromelain, BSA, laccase, lysozyme and ovalbumin. Meanwhile, the proteins with non-competitive behaviour for MA-enantiomers were the Cyt C, HSA, Hexokinase II, Amylase, GFP, Ovalbumin and Phycoerythrin. Table 9 depictes the complete list of proteins studied. The ideal protein should present a competitive behaviour (similar bidding site for both MA enantiomers in protein structure), where only one of the MA enantiomers will present more affinity and strong interactions with the amino acids residues from the protein, allowing an efficient enantioseparation. According to computational screening performed, it was decided to analyse in more detail two proteins: BSA (competitive behaviour) and Cyt C (non-competitive). Both proteins were already extracted using ABS[78], [80], [123], those being thus the most appropriate to prove the ability of the selected proteins using molecular docking as chiral selectors for MA enantiomers. All data are discriminated in Appendix B.

Table 9 - Enantioselectivity of proteins analysed with AutoDock Vina.

Protein (PDB file)	**Enantioselective behaviour**
Cyt C (1HRC)	Non-competitive
BSA (4F5S)	Competitive
HSA (1E71)	Non-competitive
Bromelain (1W0Q)	Competitive
Hexokinase II (1IG8)	Non-competitive
Amylase (1UA7)	Non-competitive
GFP (4KW4)	Non-competitive
Laccase (1KYA)	Competitive
Lysozyme (4YM8)	Competitive
Ovalbumin (1OVA)	Competitive
Phycoerythrin (1EYX)	Non-competitive

Table 10 shows the results for the best binding pose Cyt C docking with the lowest absolute value of affinity (kcal.mol^{-1}) for Cyt C with MA-enantiomers (docking affinities, interacting amino acids residues, type of interaction and geometry distance (Å) of MA-enantiomer individually). The best binding pose and type of interaction of Cyt C with MA-enantiomers are displayed in Figure 11.

Table 10 – Docking affinity energy and interacting amino acids predicted by AutoDock Vina for Cyt C- MA enantiomers.

Cytochrome C (PDB:1HRC)	Affinity (kcal.mol^{-1})	Interacting amino acids	Type of interaction	From	To	Distance (Å)
***R*-MA**	-5.0	Tyrosine67	Hydrogen bond	Tyr67	*R*-MA	3.88
			Hydrophobic	Tyr67	*R*-MA	5.23
		Leucine32		*R*-MA	Leu32	5.19
		Leucine35		*R*-MA	Leu35	5.18
***S*-MA**	-4.8	Threonine19	Hydrogen bond	*S*-MA	Thr19	2.61
		Alanine15		*S*-MA	Ala15	2.86
		Phenilalanine10	Hydrophobic	Phe10	*S*-MA	4.82
		Alanine15		*S*-MA	Ala15	4.75
		Valine20		*S*-MA	Val20	5.30

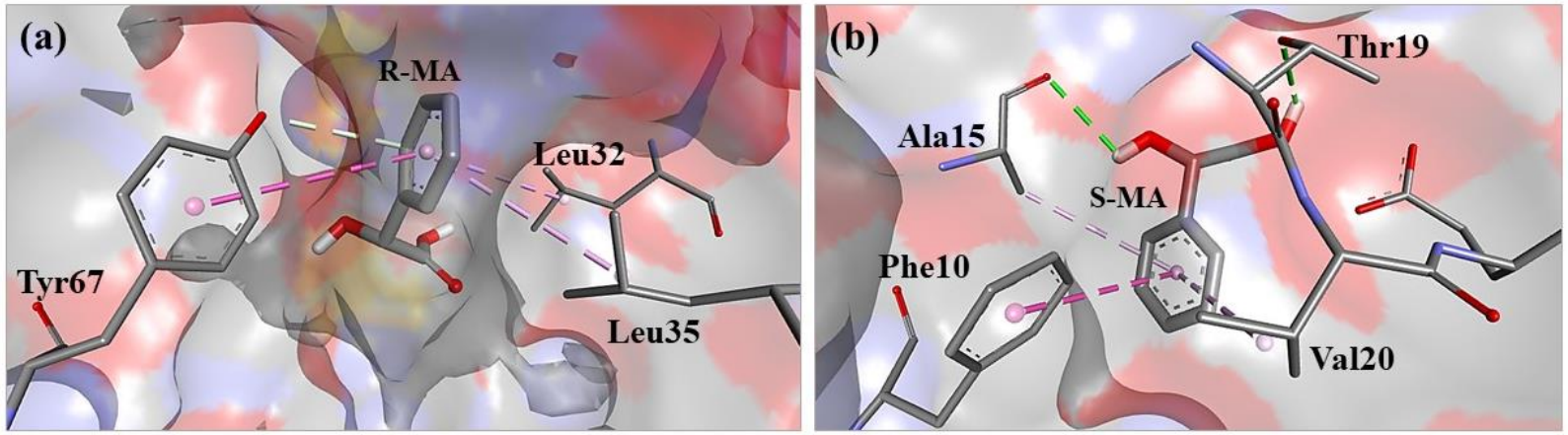

Figure 11 - The Cyt C docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for Cyt C with (a) *R*-MA and (b) *S*-MA (------) hydrogen bond, (------) π-σ bond, (------) π-alkyl bond and (------) π – donor hydrogen bond).

The results revealed that MA-enantiomers present different binding sites at Cyt C surface. The docking affinity for *R*-MA (-5.0 kcal.mol^{-1}) is higher compared to *S*-MA (-4.8 kcal.mol^{-1}). The *R*-MA interact with three amino acids, namely Leu32, Leu35 and Tyr67 (by hydrogen bond and hydrophobic interactions). However, *S*-MA shown ability to bind to 4 amino acids (Phe10, Ala15, Thr19 and Val20) also by hydrogen bond and hydrophobic interactions. Analysing individually the interactions, *S*-MA establish more favourable interactions than *R*-MA. The interaction distance must be below 5Å to be considered a viable interaction. Thus, in presence of *S*-MA, the protein-enantiomer complex is more probable. The interaction of *S*-MA suggests that this enantiomer would be more capable of forming a protein-enantiomer complex, since it has four chemical bonds bellow 5 Å and the *R*-MA only one. The type of interaction that could be promoted by MA-enantiomers is also important to highlight. According to the interaction distance, the rank of possible interactions follows the order: hydrogen bond > ionic interaction > hydrophobic interaction > van der Waals interactions. In Cyt C-*S*-MA complex, three hydrophobic interactions and two hydrogen bond interactions were predicted by molecular docking. On the other hand, Cyt C-*R*-MA complex is based in one hydrogen bond in Tyr67 residues.

Since both MA-enantiomers present different binding sites on Cyt C surface, the protein studied seems to be inefficient for enantioselective separations of MA-enantiomers. On the other hand, BSA displays an opposite behaviour. The docking pose of BSA-MA enantiomers with the lowest absolute value of affinity (kcal.mol^{-1}) are displayed in Figure 12. Docking affinities, interacting amino acids residues, type of interaction and geometry distance (Å) of MA-enantiomer individually and BSA are also shown in Table 11.

Table 11- Docking affinity energy and interacting amino acids predicted by AutoDock Vina for BSA-MA enantiomers.

BSA (PDB:4F5S)	Affinity (kcal.mol^{-1})	Interacting amino acids	Type of interaction	From	To	Distance (Å)
***R*-MA**	-5.0	Arginine208	Hydrogen bond	Arg208	*R*-MA	3.16
		Leucine346		*R*-MA	Leu346	2.07
			Hydrophobic	*R*-MA	Leu346	4.86
		Lysine350		*R*-MA	Lys350	5.47
		Valine481		*R*-MA	Val481	4.10
***S*-MA**	-4.8	Arginine208	Hydrogen bond	Arg208	*S*-MA	3.19
		Leucine346		*S*-MA	Leu346	2.14
		Alanine209	Hydrophobic	*S*-MA	Ala209	4.69
		Alanine212		*S*-MA	Ala212	5.03
		Leucine346		*S*-MA	Leu346	4.51
		Valine481		*S*-MA	Val481	4.46

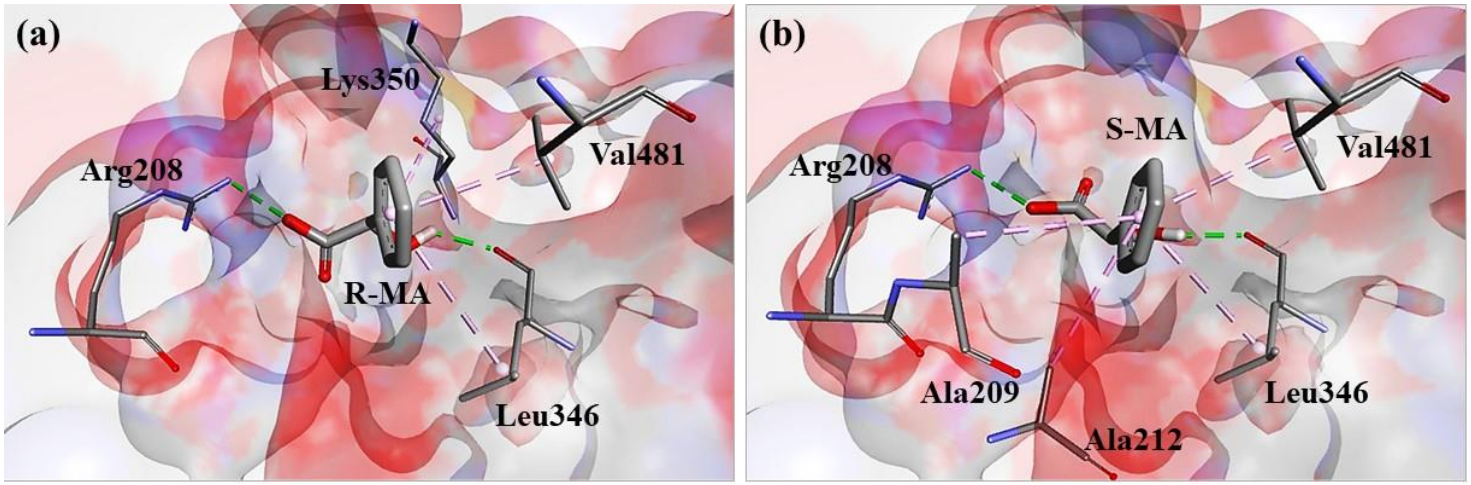

Figure 12 - The BSA docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for BSA with (a) *R*-MA and (b) *S*-MA (⁻⁻⁻⁻) hydrogen bond and (⁻⁻⁻⁻) π-alkyl bond.

The results of BSA-MA enantiomers depicted in Figure 12 show that both MA-enantiomers present similar binding sites to the BSA-surface. MA-enantiomers interact with Arg208, Leu346 and Val481. In fact, BSA was already applied as chiral selector in chromatography separation processes [107], [110], [112]. The complex formed between BSA-MA enantiomers presents the same trend on binding affinity complexes formed of Cyt C-MA enantiomers: -5.0 kcal.mol^{-1} for *R*-MA and -4.8 kcal.mol^{-1} for *S*-MA. However, BSA-MA enantiomers exhibit the enantioselective behaviour.

In presence of both MA-enantiomers it was observed four common interactions with amino acids residues: two hydrogen bonds with Arg208 and Leu346 and two hydrophobic interactions with Leu346 and Val481. Besides the similar interactions, individually *S*-MA enantiomer present two hydrophobic interactions with Ala209 and Ala212 and *R*-MA enantiomer display one hydrophobic interaction (Lys350). Therefore, these results suggest that the *R*-MA enantiomers are more able to establish enantioselective interactions with BSA surface. Even, the interactions distances are lower in *R*-MA than *S*-MA, which seems to support that the BSA-*R*-MA complex will occur more easily than the BSA-*S*-MA complex.

In summary, the results obtained indicate that in presence of BSA, MA-enantiomers will present similar binding sites on the protein surface showing the ability of BSA to promote an enantioseparation process. In order to validate the computational screening using AutoDock Vina, in next section the use of Cyt C and BSA as chiral selectors was tested regarding the use of several ABS composed of different phase-forming agents.

3.1.2. Cyt C as chiral selector in ABS

Cyt C is a hemeprotein associated with the inner membrane of the mitochondrion, being an essential component of the electron transport chain.[188] Cyt C is commonly obtained from equine heart and it is composed of 104 amino acids with ~12 kDa. This protein is normally used as a model protein for molecular evolution.[189] It was already used as a chiral selector in CEC by being incorporated in a stationary phase to separate an enantiomeric mixture of racemic amino acids. [190]

The mixture points used in ABS preparation are described in Table 6 (Chapter 2, section 2.2.1). Firstly, the partition behaviour of Cyt C and each one of the mandelic acid enantiomers was performed separately. The results obtained for the partition of Cyt C in different ABS, namely polymer-polymer, polymer-salt and salt-IL, are reported in

Figure 11a. The E.E.$_{Cyt\ C}$, E.E.$_{R/S\text{-}MA}$ and *e.e.* values were calculated to the phase where Cyt C was more concentrated and all detailed data is presented in Appendix C.

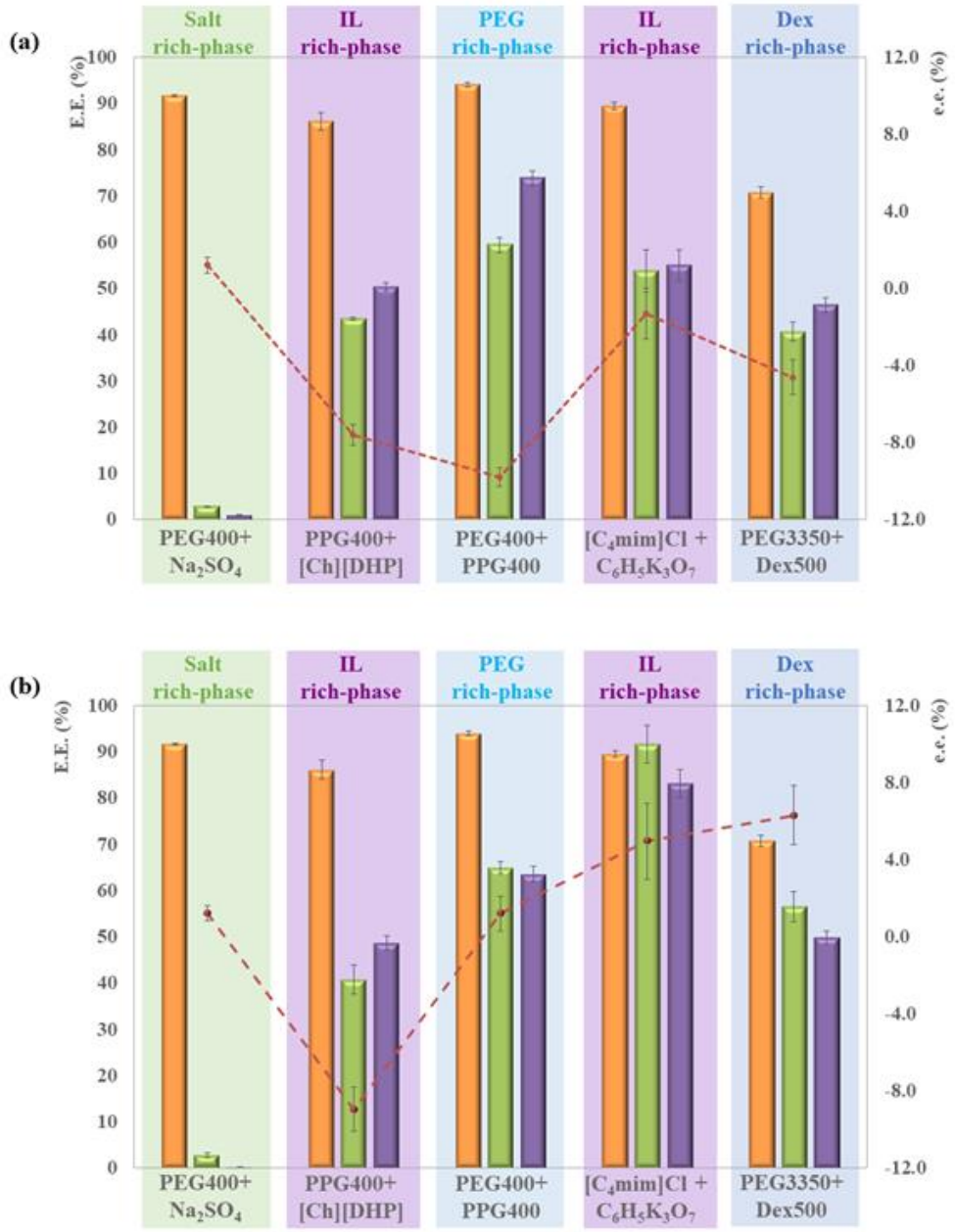

Figure 13 - Extraction efficiencies of Cyt C (E.E.$_{CytC}$, ■), *R*-MA (E.E.$_{R\text{-}MA}$ ■) and *S*-MA (E.E.$_{S\text{-}MA}$,■) along with the enantiomeric excess (e.e., ●) obtained for the ABS studied. (a) partition studies in ABS with MA racemate and Cyt C performed separately. (b) enantioseparation studies in ABS with MA racemate and Cyt C performed together.

The protein's partition is a complex process that depends on the interactions between the protein and the phase formers of the system. Many are the factors that affect the protein partition in any ABS, namely polymer's molecular mass, size and concentration, salt's ionic strength and medium pH. [54] The systems tested perform

according to the following decreasing order in what regards the concentration of Cyt C in a single phase (according to the $E.E._{Cyt\ C}$):

PEG400+PPG400 ($E.E._{Cyt\ C}$ = 93.99 ± 0.5%) > PEG400+Na_2SO_4 ($E.E._{Cyt\ C}$ = 91.66 ± 0.22%) > [C_4mim]Cl+$C_6H_5K_3O_7$ ($E.E._{Cyt\ C}$ = 89.44 ± 0.75%) > PPG400+[Ch][DHP] ($E.E._{Cyt\ C}$ = 86.13 ± 2.01%) > PEG3350+Dex500 ($E.E._{Cyt\ C}$ = 70.74 ± 1.24%)

In the systems composed of polymer+salt/IL, the protein partitions preferentially for the most hydrophilic Na_2SO_4/[Ch][DHP]-rich phase, with a $E.E._{Cyt\ C}$ of 91.66 ± 0.22% and 86.13 ± 2.01%, respectively. Moreover, and since PBS is used to keep the pH of the systems at pH 7.4, positively charged Cyt C is present (p.I. = 10-10.5).[191] This could explain the partition of the protein to the Na_2SO_4/[Ch][DHP]-rich phase since the amino acids on Cyt C's surface will establish electrostatic interactions with the SO_4^{2-}/$[DHP]^-$ anions.

For the systems composed of two polymers, Cyt C is mainly present in the most hydrophilic (PEG400-rich or Dex500)-rich phase. When comparing the chemical structure of PPG400 and PEG400, PPG is more hydrophobic, since it is composed of an extra methyl group. Dextran, being a glucan, is more hydrophilic than PEG. Thereby, the protein partition seems to be driven by the polarity of the phases in polymer+polymer-based ABS.

In the case of [C_4mim]Cl+$C_6H_5K_3O_7$ system, the IL-rich phase is the phase enriched in Cyt C ($E.E._{Cyt\ C}$ of 89.44 ± 0.75%), in spite of being the most hydrophobic phase. In this case, the Cyt C partition is driven by specific interactions with the IL. The protein is positively charged and will establish electrostatic interactions with the Cl^- anion. Moreover, π-π interactions between the aromatic ring of the $[C_4mim]^+$ cation and the aromatic amino acid residues of the protein may also occur.

In the same ways to the partition behaviour of the protein, the partition of MA enantiomers may also be influenced by several interactions occurring between the MA and the ABS phase formers. For the extraction of the enantiomers, the best order to extract the mandelic acid to one phase is (according to the $E.E._{R\text{-}MA/S\text{-}MA}$ values):

PEG400+PPG400 > [C_4mim]Cl+$C_6H_5K_3O_7$ > PPG400+[Ch][DHP] > PEG3350+Dex500 > PEG400+Na_2SO_4

For the systems composed of polymer+salt/IL, the PEG400+Na_2SO_4 ABS performs better at extracting both enantiomers to the PEG-rich phase, with a very low amount of MA migrating to the salt-rich phase, as represented in Figure 13a. This is a consequence of the higher hydrophobicity of MA, as indicated by the logarithmic function of its

octanol-water partition coefficient (log Kow) of 0.90.[192] However, in both IL-based ABS, a similar partition between the two phases is observed and a relationship between the polarity of the phases and the extraction efficiency of MA is not observed. This indicates that ILs are able to interact specifically with MA enantiomers.

In the case of polymer-polymer systems, the PEG3350+Dex500 system induces an almost equivalent partition of the enantiomers to both phases ($E.E._{R\text{-}MA}$ = 40.69 ± 2.02% and $E.E._{S\text{-}MA}$ = 46.40 ± 1.56%), while PEG400+PPG400-based ABS yields a more significant partition to the more hydrophilic PEG-rich phase ($E.E._{R\text{-}MA}$ = 59.44 ± 1.62% and $E.E._{S\text{-}MA}$ = 73.82 ± 1.68%). The difference in the hydrophobicity/hydrophilicity of the phases is more important for PEG400+PPG400 than for PEG3350+Dex500, what induces a more significant partition of the enantiomers to one phase.

It should be noted that, since no chiral selector was added to these systems (with the exception for the Dex500-based ABS), no significant enantiomeric separation was expected as shown by the low *e.e.* values obtained (-9.8 ± 0.5% < *e.e.* < 1.2 ± 0.4%). To develop a system for chiral resolution, a system where the protein and the enantiomers partition to distinct phases may be the best option. The introduction of Cyt C may favour chiral interactions with only one enantiomer manipulating its partition to the other phase. In this context, the system composed of PEG400 and Na_2SO_4 seems to be the best option because Cyt C partitions to the salt-rich phase and the enantiomers to the polymer-rich layer. In this sense, and having studied the partition of the protein and both enantiomers separately, it was then proceed the test using the protein and the enantiomers simultaneously in the same systems to validate their real chiral resolution. The results obtained for the E.E. and *e.e.* are shown in Figure 13b and all data are discriminated in Appendix C.

The introduction of Cyt C in the systems here studied was shown to influence the partition of the enantiomers. The enantioseparation aptitude of the Cyt C containing ABS follows the decreasing order (based on the *e.e.* values):

PPG400+[Ch][DHP] (*e.e.* = -9.0 ± 1.2%) > PEG3350+Dex500 (*e.e.* = 6.3 ± 1.6%) > [C_4mim]Cl+$C_6H_5K_3O_7$ (*e.e.* = 5.0 ± 2.0%) > PEG400+PPG400 (*e.e.* = 1.9 ± 0.9%) >PEG400+Na_2SO_4 (*e.e.* = 1.2 ± 0.4%)

In general, and as corroborated by the molecular docking results, no significant improvements were caused by the presence of Cyt C in the ABS screened. PPG400+[Ch][DHP]+Cyt C-based ABS yielded an *e.e.* = -9.0 ± 1.2% – the best result achieved – whereas, PEG400+Na_2SO_4+Cyt C failed in the separation of the enantiomers

(*e.e.* = 1.2 ± 0.4%). Not only the inability of the protein to act as chiral selector, but also the salting-out power of Na_2SO_4 may contribute for this result. The introduction of Cyt C in the $[C_4mim]Cl+C_6H_5K_3O_7$ system increased the presence of *R*-MA in the IL rich-phase up to *e.e.* = 5.0 ± 1.0%. By now, the choice of strong salting-out agents, as Na_2SO_4 or $C_6H_5K_3O_7$, seems to negatively affect the enantioseparation.

Polymer+polymer systems dismiss the use of salting out agents as they are formed by the incompatibility of the polymers in aqueous solution. In the PEG400+PPG400 system, no significant enantioselectivity was observed (*e.e.* = 1.2 ± 0.9%), opposing to the other polymer-polymer system (PEG3350+Dex500) where the *e.e.* varied from -4.8 ± 0.9% (for *S*-MA) to 6.3 ± 1.6% (for *R*-MA). Dextran, being a glucan, may display some chirality and thus, induce some enantioseparation without the presence of Cyt C. The presence of Cyt C, by its side, plays a certain role in this specific ABS as it intensifies the presence of *R*-MA opposing to *S*-MA. Furthermore, PEG3350+Dex500 has a more tenuous difference between the phases' polarities allowing a better enantioseparation than the PEG400+PPG400 ABS.

These results indicate the importance of choosing the adequate phase formers and tuning their properties, namely salting-out of the salt and hydrophilic/hydrophobic differences of the phases, to achieve better enantioseparations.

3.1.3. BSA as chiral selector in ABS

BSA is a bovine serum albumin responsible by the blood transport in the species *Bos taurus*. It is constituted by 583 amino acids and the molecular weight is 66.5 kDa. Beyond the BSA low cost, which is a useful feature for the economic viability of the chiral ABS development, its ability to act as chiral selector was already shown (Chapter 1.2, Tables 1 and 2). Techniques such as chromatography [102,109], membrane-based separations[104,112] and ABS [78] are some examples.

Some ABS reported in literature show the outstanding ability to completely extract BSA into a single phase.[173,179] In this context, in this section, the types of ABS tested were chosen based on the extraction results reported in literature: for the IL/salt and IL+polymer systems studied, the $E.E._{BSA}$ results used were those reported by Pereira *et al.*[175] and Quental *et al.*[181]; and for the polymer+ (D)-sucrose, the results of BSA extraction were used from the results already reported by Taha *et al.* [168]. In order to enlarge the systems screened, the $E.E._{BSA}$ for the ABS composed of

PPG400+Na_2SO_4, PPG400+PEG400 and PEG3350+Dex500 were determined in the present work to allow further enantioseparation studies. All detailed data is present in Appendix C.

The following combinations were used: PPG400+[Ch][Ac][181], PPG400+[Ch][DHCit][181], PPG400+Na_2SO_4[183], PPG400+(D)-Sucrose[168], PPG400+PEG400[56], PEG3350+Dex500[172], [$P_{i(444)l}$][Tos]+Citrate buffer at pH 6[175], [P_{4444}]Cl+ $K_3C_6H_5O_7/C_6H_5O_7$, at pH 6[175] and [N_{4444}]Cl+ $K_3C_6H_5O_7/C_6H_5O_7$ at pH 6[175]. The ability of systems to concentrate BSA into a single phase is ranked as follows:

[N_{4444}]Cl+$K_3C_6H_5O_7/C_6H_5O_7$ ($E.E._{BSA}$ = 100%) = [P_{4444}]Cl+$K_3C_6H_5O_7/C_6H_5O_7$ ($E.E._{BSA}$ = 100%) = [$P_{i(444)l}$][Tos]+$K_3C_6H_5O_7/C_6H_5O_7$ ($E.E._{BSA}$ = 100%) = PPG400+[Ch][Ac] ($E.E._{BSA}$ = 100%) = PPG400+[Ch][DHCit] ($E.E._{BSA}$ = 100%) = PPG400+Sucrose ($E.E._{BSA}$ = 100%) > PEG3350+Dex500 ($E.E._{BSA}$ = 89.43 ± 1.24%) ~ PEG400+PPG400 ($E.E._{BSA}$ = 90.36 ± 0.5%) >> PPG400+Na_2SO_4 ($E.E._{BSA}$ = 55.00 ± 2.55%)

Considering the pI of BSA (4.8)[193] and the pH medium used in the systems (PBS at pH = 7.4), the protein is negatively charged and electrostatic interactions between cations of IL and the amino acids on the BSA surface will be established. For the IL+salt ABS, BSA is completely extracted to the IL-rich phase. This behaviour was explained by Pereira *et al.*[175] by "a combined effect of favourable dispersive interactions, salting-out effect exerted by the citrate-based salt, and electrostatic interactions between the positively charged IL cations and the negatively charged amino acid residues at the surface of the protein". For the PPG400+[Ch]-based ILs ABS, BSA migrates to the [Ch]-rich phase as a consequence of "a delicate balance among hydrogen bonding and dispersive interactions occurring between the protein and the IL ions", as proposed by Quental *et al.*[181] The same phenomenon was verified for the partition of Cyt C in the same type of systems. Moreover, in the PPG+Na_2SO_4 system, the BSA has almost an equal partition to both phases. From the overall results for these three types of systems (IL+salt, IL+polymer and polymer+salt), it seems that ILs are able to establish specific interactions with the protein if well-designed, allowing to achieve single-step complete extractions.

In both polymer+polymer systems, BSA partitions preferentially to the more hydrophilic phases, Dex500 rich- and PEG500-rich phases. Similarly, in PPG400+(D)-sucrose, a complete partition of BSA to the most hydrophilic sugar-rich phase is

observed. This scenario indicates that BSA partition is dominated by the hydrophilicity/hydrophobicity of the phases in polymer+polymer and polymer+sugar systems, as also observed when Cyt C was used.

For the extraction of the enantiomers, the order to extract MA enantiomers to one phase is the following (according to the $E.E._{R\text{-}MA/S\text{-}MA}$ values):

$[N_{4444}]Cl$+$K_3C_6H_5O_7/C_6H_5O_7$ > PPG400+[Ch][Ac] > PPG400+Na_2SO_4 > $[P_{4444}]Cl$+$K_3C_6H_5O_7/C_6H_5O_7$ > $[P_{i(444)1}][Tos]$+$K_3C_6H_5O_7/C_6H_5O_7$ > PPG400+[Ch][DHCit] > > PEG3350+Dex500 > PPG400+(D)-sucrose > PEG400+PPG400

In PPG400+Na_2SO_4 and PPG400+(D)-sucrose system, both enantiomers migrated preferentially to the PPG400 rich-phase as a result of the hydrophobic character of MA (translated by log Kow of 0.90).[192] Again, when ILs are used in polymer-based ABS, it is possible to observe an inversion of this behaviour. PPG400+[Ch][Ac] extracts both MA enantiomers to the IL-rich phase, which is more hydrophilic ($E.E._{R\text{-}MA}$ of 99.45 ± 1.32% and $E.E._{S\text{-}MA}$ 93.75 ± 3.62%). This result indicates that specific interactions between the MA and [Ch][Ac] are taking place.

All three IL-citrate buffer systems had values of $E.E._{MA}$ above 80%, being the $[N_{4444}]Cl$ found to be the best IL to extract both enantiomers to the most hydrophobic IL-rich phase ($E.E._{R\text{-}MA}$ of 95.84 ± 2.21% and $E.E._{S\text{-}MA}$ of 91.18 ± 3.19%). When comparing $[P_{4444}]Cl$ and $[N_{4444}]Cl$ performance, the last is more hydrophilic[182,194] and the MA enantiomers will have more affinity to the $[N_{4444}]Cl$. Once more, the MA extraction is driven by specific interactions established between the enantiomers and the IL in the system.

In the case of polymer+polymer systems, as already described in the previous section, a delicate balance between the phases' hydrophobicity-hydrophilicity must be achieved to tune the MA partition.

It should be highlighted that the ABS formed by PPG400 and (D)-Sucrose without BSA yielded promising enantiomeric excesses of -12.3 ± 0.5%, due to the presence of a chiral selector also as phase former. This system will be object of further optimization in section 3.2.

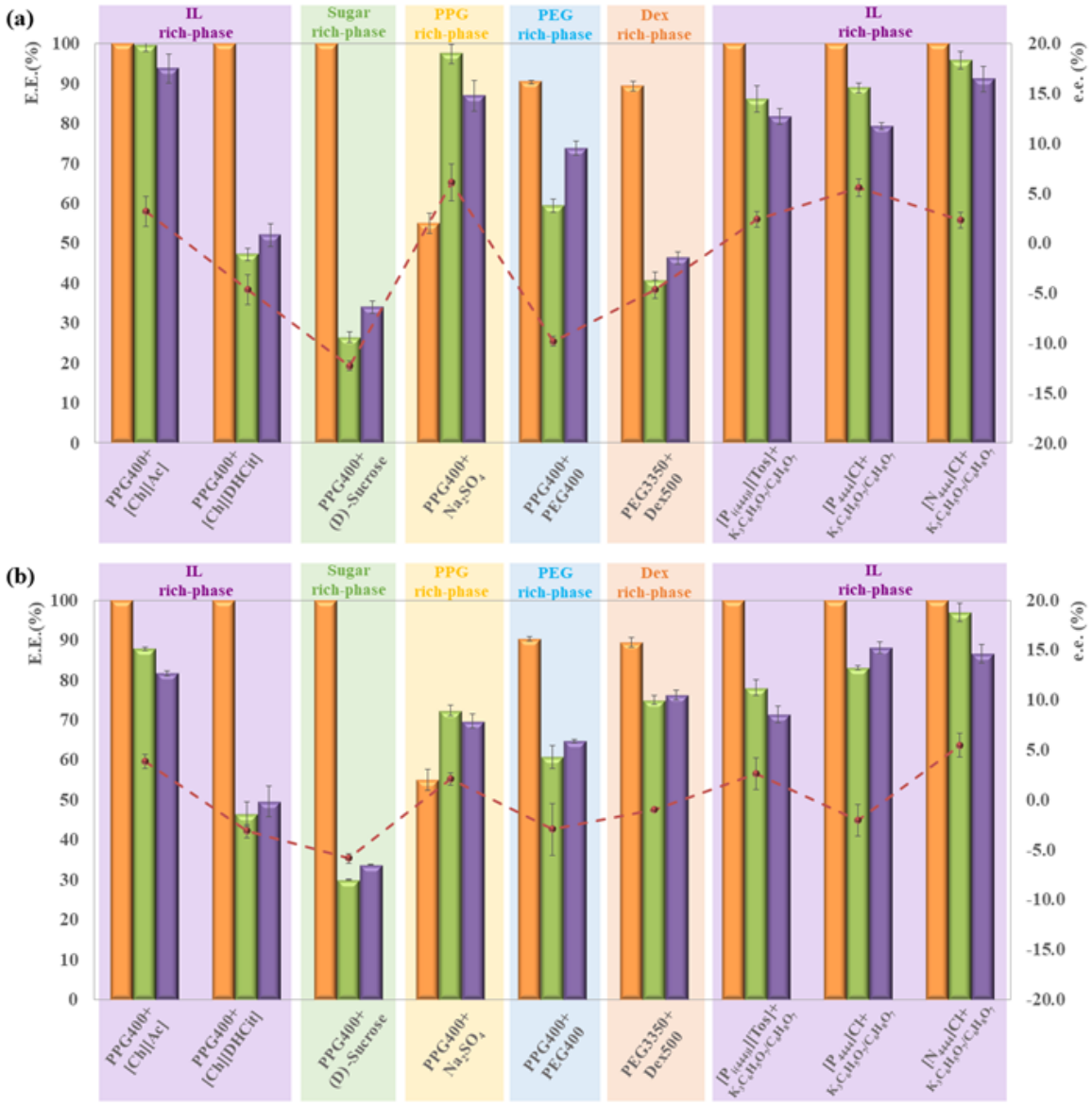

Figure 14 - Extraction efficiencies of BSA (E.E.$_{BSA}$, ■), *R*-MA (E.E.$_{R\text{-}MA}$, ■) and S-MA (E.E.$_{S\text{-}MA}$, ■) along with the enantiomeric excess (e.e., ●) obtained for the ABS studied. (a) partition studies in ABS with MA racemate and BSA performed separately. (b) enantioseparation studies in ABS with MA racemate and BSA performed together.

PPG400+(D)-Sucrose is the ABS presenting the most significant difference in the migration tendencies for the protein and the enantiomers, seeming to be the best option for the development of novel chiral ABS containing BSA for MA enantioseparation. As for the previous studies with Cyt C, it is necessary to test the protein and the enantiomers simultaneously in the same systems to validate the real chiral resolution ability of these systems. The results obtained for the E.E. and *e.e.* in the BSA-based systems are shown in Figure 14b. It can be concluded that after introducing BSA in the ABS, there were some changes in the extraction and enantioseparation of MA enantiomers. Based on the molecular docking results, BSA should have more affinity to

the *R*-MA and this way, it will form a complex with this *R*-MA more easily, leaving *S*-MA in the other phase. The ABS enantioseparation ability can be organized as follows (according the *e.e.* values):

PPG400+(D)-sucrose (*e.e.* = -5.9 ± 0.5%) > $[N_{4444}]Cl+K_3C_6H_5O_7/C_6H_5O_7$ (*e.e.* = 5.5 ± 1.2%) > PPG400+[Ch][Ac] (*e.e.* = 3.8 ± 0.7%) > PPG400+[Ch][DHCit] (*e.e.* = -3.1 ± 0.6%) ~ PEG400+PPG400 (*e.e.* = -3.0 ± 0.6%) > $[P_{i(444)I}][Tos]+K_3C_6H_5O_7/C_6H_5O_7$ (*e.e.* = 2.6 ± 1.6%) > $[P_{4444}]Cl+K_3C_6H_5O_7/C_6H_5O_7$ (*e.e.* = 2.1 ± 1.6%) = PPG400+Na_2SO_4 (*e.e.* = 2.1 ± 0.6%) > PEG3350+Dex500 (*e.e.* = -1.0 ± 0.1%)

The best system for MA enantioseparation was the PPG400+(D)-sucrose+BSA. However, BSA decreased almost two times the enantiomeric excess. By introducing BSA, there was an increase in *R*-MA amount in the (D)-sucrose-rich phase, what is in accordance with the molecular docking results. Contrarily, in the ABS composed of $[P_{4444}]Cl$ and citrate buffer systems, the presence of BSA induced a slightly higher prevalence of *S*-MA in the IL-rich phase. This result may indicate that an appropriate choice of the phase formers is important for chiral recognition to occur. For instance, in this specific case, other interactions between the IL, the citrate salt, the protein and the MA may play a role. For the remaining systems, no significant effects were noticed.

3.1.4. Can molecular docking be used to choose proteins as chiral selectors in ABS? - Cyt C *versus* BSA

In order to infer on how molecular docking can be used to choose proteins as chiral selectors in ABS, it is important to compile all the results and compare the performance of distinct ABS containing Cyt C and BSA. For that, the ABS tested (PPG400+PEG400 and PEG3350+Dex500) for both proteins were compared along with the ones yielding the highest enantiomeric excesses, according to Figure 15.

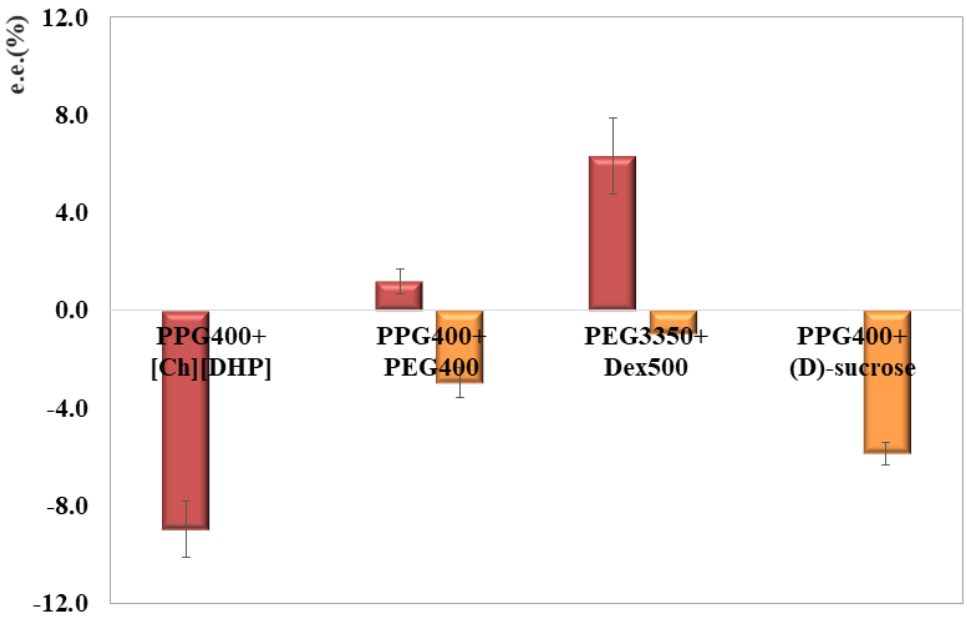

Figure 15 – Enantiomeric excesses of MA with BSA (*e.e.*$_{BSA}$, ■) and Cyt C (*e.e.*$_{Cyt\ c}$, ■) as chiral selectors in representative ABS.

It can be concluded that the efficiency of a protein as chiral selector is dependent on the ABS phase formers and that distinct proteins will act differently in distinct systems. In the PPG400+PEG400 system, BSA and Cyt C perform similarly (-3.0 ± 0.6% *versus* 1.9 ± 0.5%). Instead, when analysing the PEG3350+Dex500 system, Cyt C was shown to be a better chiral selector than BSA (6.3 ± 1.6% versus -1.0 ± 0.1%). The most significant values were achieved in PPG+[Ch][DHP]+Cyt C with *e.e.* of -9.0 ± 1.2% and PPG+(D)-sucrose+BSA with *e.e.* of -5.9 ± 0.5%. From the results gathered, it is possible to conclude that molecular docking does not seem to be the most adequate predicting tool for proteins as chiral selectors in ABS. Yet, further investigation on this aspect is needed. Molecular docking allows gaining insight into the interactions occurring between proteins and enantiomers and it can be used from a molecular perspective to explain the interactions occurring between BSA/Cyt C and the MA enantiomers. This bioinformatic tool is very useful for other types of studies, namely in drug design [149,154] and biocatalysis [146,147,152] (see Table 4). However, during this work, it was shown that the enantioseparation in ABS is largely influenced by the phase formers. This aspect can be identified as the main factor limiting the success of using molecular docking as predicting tool, since AutoDock Vina can only visualize the interactions between the protein and the small molecule, dismissing the interactions from surrounding molecules. In Figure 16, it is shown the workflow for the dissertation.

During the tests, the polymer-sugar system with the best result to enantioseparation without protein, suggested another path to explore: a phase former with the capacity of chiral selector.

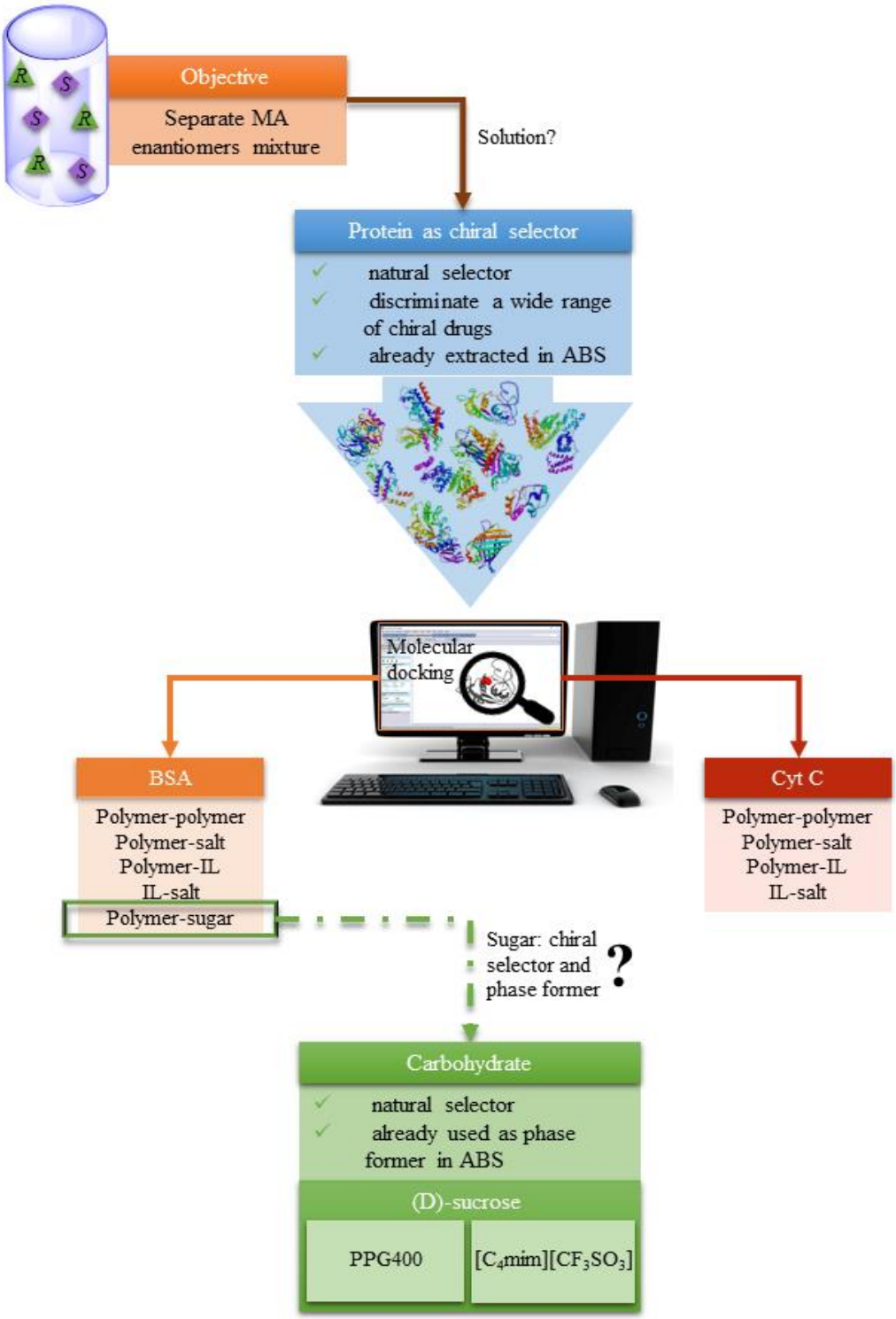

Figure 16 – Scheme of work-flow to explain the used of ABS with (D)-sucrose.

3.2. Sucrose as chiral selector and phase former in ABS for MA enantioseparation

As mentioned above, PPG400+(D)-sucrose system revealed enhanced enantioseparation ability when compared to the poor performance of proteins. Under this scenario, the use of sucrose as phase former and chiral selector was objective of further investigation. Sucrose is a naturally occurring carbohydrate, consisting of a disaccharide of glucose and fructose. The potential of sucrose as chiral selector is well-known, for instance in the determination of the enantiomeric composition of a D-Phe/L-Phe mixture by UV-Vis spectroscopy.[161] The potential of sucrose as ABS phase former is recognized, when combined with different ILs[68,194] or polymers.[168]

Two distinct types of ABS were included in this study: PPG400+(D)-sucrose and $[C_4mim][CF_3SO_3]$+(D)-sucrose. A common extraction point selected from the phase diagrams reported in literature [68,167] was adopted for these two systems, allowing the direct comparison (40 wt% (D)-sucrose + 20 wt% PPG400/$[C_4mim][CF_3SO_3]$). The extraction efficiencies and enantiomeric excesses toward the sugar-enriched phase are presented in Figure 17 and all detailed data is presented in Appendix C. When comparing different systems, the extraction efficiencies and enantiomeric excesses for *S*-MA are similar, suggesting that (D)-sucrose is the phase former controlling the MA migration and both PPG400 and $[C_4mim][CF_3SO_3]$ play a minor role. When compared to the systems including proteins, these systems show improved performance and lower operational complexity, once (D)-sucrose encompasses two functions (phase former and chiral selector).

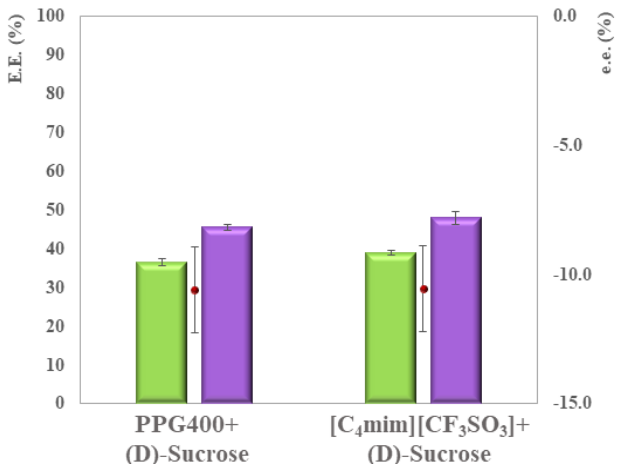

Figure 17 - Extraction efficiencies of *R*-MA (E.E.$_{R\text{-}MA}$ ■) and *S*-MA (E.E.$_{S\text{-}MA}$,■) along with the enantiomeric excess (*e.e.*, ●) obtained for the (D)-sucrose-based ABS studied.

Given the promising results obtained and to maximize the enantioseparations afforded by these systems, the ABS composition was optimized. For that, distinct mixture points were selected at the biphasic region of the phase diagram (26 wt% PPG400+26 wt% (D)-sucrose, 20 wt% PPG400 + 40 wt% (D)-sucrose and 30 wt% PPG400 + 30wt% (D)-sucrose). The respective results are depicted in Figure 18 and indicate a considerable influence of the extraction point on both the MA extraction efficiency and enantiomeric excess. Extraction points located closer to the binodal curve yield phases with higher water contents; those located far induce the formation of phases richer in either the polymer (top) or sucrose (bottom). It can be concluded that lower water contents prompt the enantioseparations, although slightly minimizing the extraction efficiencies. This result indicates that the extraction of MA in PPG400/(D)-sucrose system is driven by the hydrophobicity/hydrophilicity of the phases. The same findings were provided by Zafarani-Moattar *et al.* for paracetamol extraction in polymer/ILs systems [196]. Regarding enantioseparation, as soon as the (D)-sucrose becomes richer in the sugar, more interactions will occur between *S*-MA and (D)-sucrose, increasing the enantiomeric excess up to 2-fold.

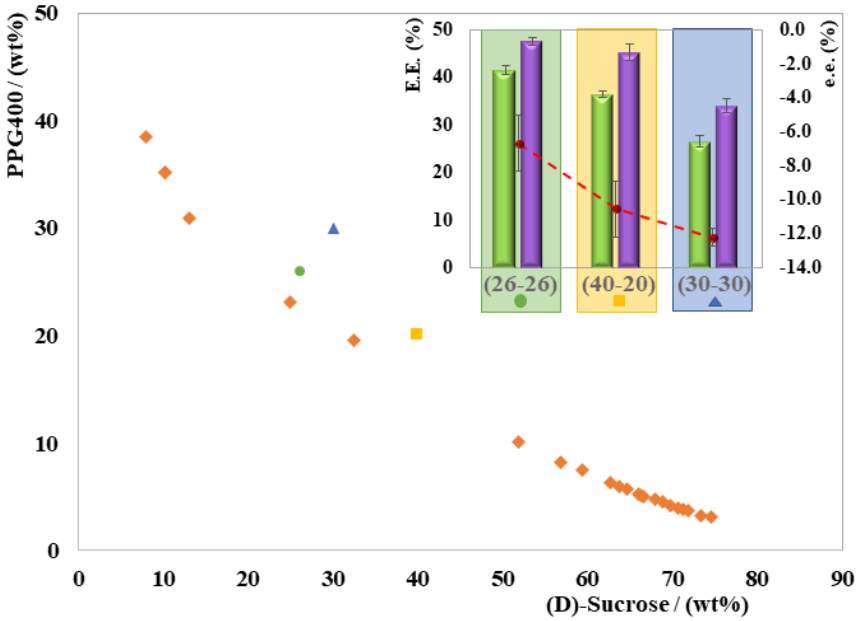

Figure 18 – Evaluation of the mixture point influence on the extraction efficiencies of *R*-MA (E.E.$_{R\text{-}MA}$ ■) and *S*-MA (E.E.$_{S\text{-}MA}$,■) and the enantiomeric excess (*e.e.*, ●) in the (D)-sucrose-based ABS: phase diagram data (♦); mixture points at 26 wt%+26 wt% (●),40 wt%+20 wt% (■) and 30 wt%+30 wt% of (D)-sucrose and PPG400, respectively (▲).

4. Conclusions and Future Work

4.1. Conclusions

In this work, different approaches to separate MA enantiomers were attempted. One was based on the use of proteins as chiral selectors in ABS and the other was related with the use of D-sucrose as both phase former and chiral selector in ABS.

A bioinformatic tool, AutoDock Vina, was used to predict the interactions between several proteins and MA enantiomers. With this computational screening, it was expected to select ideal proteins as chiral selectors in ABS, avoiding experimental trial-error screenings and thus saving money and time. The role of proteins as chiral selectors in ABS was shown to be dependent on both the protein used (Cyt C or BSA) and on the type of phase formers selected (polymer+salt, polymer+polymer, polymer+IL, IL+salt and polymer+sugar). The best enantioseparation results were obtained for PPG400+[Ch][DHP]+Cyt C (*e.e.* = -9.0 ± 1.2%) and for PPG400+(D)-sucrose+BSA (*e.e.* = -5.9 ± 0.5%). Although the obtained enantioseparation abilities are somehow limited, a balance of interactions occurring between the protein, enantiomers and phase formers influences both the partition and enantioseparation within the ABS tested. However, the molecular docking screening failed to fit the experimental results obtained likely due to the effect that the phase formers seem to exert. Still, AutoDock Vina can be useful to understand the interactions in the complexes protein-enantiomer formed and further studies may be valuable.

During this thesis, the most performant ABS achieved was composed of PPG400 and (D)-sucrose system. After an optimization of the component paired with (D)-sucrose (PPG400 or [C_4mim][CF_3SO_3]) and the mixture point utilized, a maximum *e.e.* of -12.3 ± 0.5% was obtained. Lower water contents in the ABS were shown to favour enantiorecognition.

4.2. Future Work

Further optimization of the ABS here screened must be performed. Most importantly those composed of (D)-sucrose but also the most promising protein-based ABS, should be studied regarding extraction points, protein type/concentration, enantiomers concentration and pH. Moreover, an extended panoply of ABS and proteins should be investigated to confirm whether molecular docking is adequate or not.

The system composed of PPG400+(D)-sucrose, being of simpler nature and composed of cheap chemicals, could easily be operated in continuous mode (using

centrifugal partition chromatography or counter-current chromatography), which could enable full separation of the enantiomers.

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Appendix

A. Calibration curves of *R*- and *S*-mandelic acid

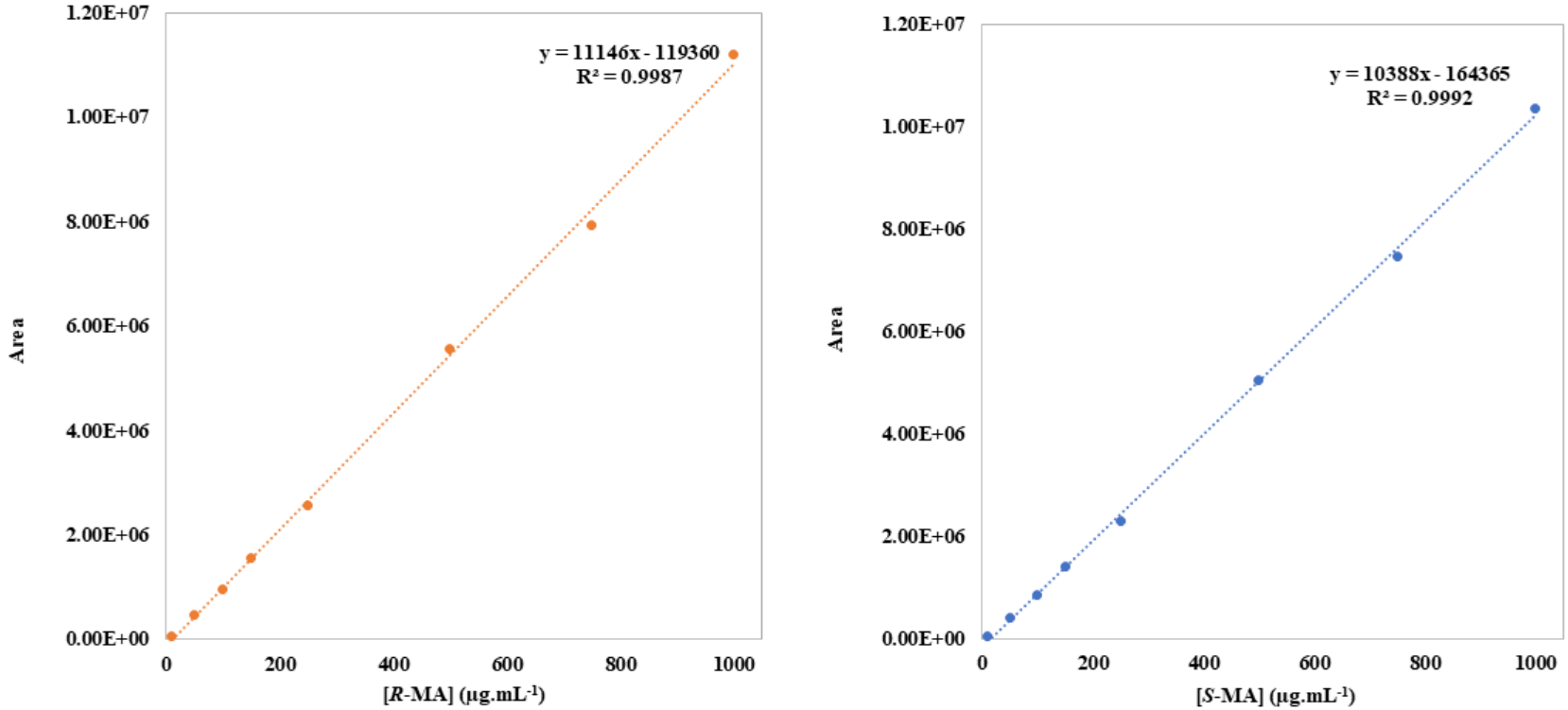

Figure A.1 - Calibration curves of *R*-MA (●) and *S*-MA (●), respectively.

B. Molecular docking

Table B.1 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for amylase- MA enantiomers.

α-amylase (PDB: 1UA7)	**Affinity (kcal.mol^{-1})**	**Interacting amino acids**	**Type of interaction**	**From**	**To**	**Distance (Å)**
R-MA	-5.0	Glutamate208	Hydrogen bond	*R*-MA	Glu208	2.57
		Aspartate269		*R*-MA	Asp 269	1.79
			Electrostatic	Asp269	*R*-MA	3.71
		Tyrosine62	Hydrophobic	Tyr62	*R*-MA	5.18
S-MA	-4.8	Triptophan58	Hydrogen bond	Trp58	*S*-MA	3.18
		Aspartate274		*S*-MA	Asp274	2.95
				S-MA	Asp274	2.98
		Tyrosine59	Hydrophobic	Tyr59	*S*-MA	4.12

Table B.2 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for Bromelain- MA enantiomers.

Bromelain (PDB:1W0Q)	**Affinity (kcal.mol^{-1})**	**Interacting amino acids**	**Type of interaction**	**From**	**To**	**Distance (Å)**
R-MA	-5.4	Lysine18	Hydrogen bond	Lys18	*R*-MA	2.62
		Phenyl29		*R*-MA	Phe29	2.29
		Ser16		*R*-MA	Ser16	3.01
		Valine17		Val17	*R*-MA	2.85
		Alanine33	Hydrophobic	*R*-MA	Ala33	3.85
S-MA	-5.4	Cystosine26	Hydrogen Bond	*S*-MA	Cys26	2.54
		Threonine161		*S*-MA	Thr161	2.50
		Alanine30		Ala30	*S*-MA	2.40
		Lysine18	Hydrophobic	*S*-MA	Lys18	5.34
		Alanine33		*S*-MA	Ala33	4.94

Table B.3 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for GFP- MA enantiomers.

Green fluorescent protein (PDB:4KW4)	Affinity (kcal.mol^{-1})	Interacting amino acids	Type of interaction	From	To	Distance (Å)
R-MA	-5.1	Serine28	Hydrogen bond	Ser28	*R*-MA	2.35
		Aspartate19		*R*-MA	Asp19	2.70
			Electrostatic	Asp19	*R*-MA	4.08
		Arginine122	Hydrophobic	*R*-MA	Arg122	4.54
S-MA	-4.7	Histidine139	Hydrogen bond	His139	*S*-MA	3.02
		Valine55		*S*-MA	Val55	2.22
		Aspartate216		*S*-MA	Asp216	3.10
		Tyrosine143	Hydrophobic	Tyr143	*S*-MA	5.26

Table B.4 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for Hexokinase II- MA enantiomers.

Hexokinase PII (PDB:1IG8)	Affinity (kcal.mol^{-1})	Interacting amino acids	Type of interaction	From	To	Distance (Å)
R-MA	-4.4	Leucine387	Hydrogen bond	*R*-MA	Leu387	2.11
		Threonine35		*R*-MA	Thr35	2.09
		Arginine394		Arg394	*R*-MA	3.58
		Alanine390	Hydrophobic	*R*-MA	Ala390	3.47
S-MA	-4.6	Threonine108	Hydrogen bond	Thr108	*S*-MA	2.91
		Aspartate106		*S*-MA	Asp106	2.59
		Threonine108		*S*-MA	Thr108	2.11
		Lysine98	Hydrophobic	*S*-MA	Lys98	3.79

Table B.5 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for HSA- MA enantiomers.

Human Serum Albumin (PDB:1E7I)	Affinity (kcal.mol^{-1})	Interacting amino acids	Type of interaction	From	To	Distance (Å)
R-MA	-5.6	Valine418	Hydrophobic	Val418	*R*-MA	3.95
				Val418	*R*-MA	3.80
		Valine415		*R*-MA	Val415	5.11
		Valine426		*R*-MA	Val426	5.41
		Leucine460		*R*-MA	Leu460	4.47
			Hydrogen	*R*-MA	Leu460	2.92
				R-MA	Leu460	2.99
S-MA	-5.6	Valine23	Hydrophobic	*S*-MA	Val23	5.44
		Leucine66		*S*-MA	Leu66	5.06

Table B.6 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for Lysozyme- MA enantiomers.

Lysozyme (PDB:4YM8)	Affinity (kcal.mol^{-1})	Interacting nucleic acids	Type of interaction	From	To	Distance (Å)
R-MA	-4.4	Asparagine59	Hydrogen bond	Asn59	*R*-MA	3.11
		Tryptophan63		Trp63	*R*-MA	2.95
		Alanine107		*R*-MA	Ala107	1.97
		Aspartate52	Electrostatic	Asp52	*R*-MA	4.71
S-MA	-4.4	Aspartate101	Hydrogen bond	*S*-MA	Asp101	1.95
			Electrostatic	Asp101	*S*-MA	4.46
		Tryptophan62	Hydrophobic	Trp62	*S*-MA	3.88
				Trp62	*S*-MA	3.75

Table B.7 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for Laccase- MA enantiomers.

Laccase (PDB:1KYA)	Affinity (kcal.mol^{-1})	Interacting nucleic acids	Type of interaction	From	To	Distance (Å)
R-MA	-5.0	Phenylalanine162	Hydrophobic	Phe162	*R*-MA	4.22
		Proline391		*R*-MA	Pro391	4.96
		Histidine458	Hydrogen bond	*R*-MA	His458	2.28
S-MA	-5.0	Phenylalanine162	Hydrophobic	Phe162	*S*-MA	4.26
		Proline391		*S*-MA	Pro391	4.74
		Glycine392	Hydrogen bond	Gly392	*S*-MA	3.14

Table B.8 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for Ovalbumin- MA enantiomers.

Ovalbumin (PDB:1OVA)	Affinity (kcal.mol^{-1})	Interacting nucleic acids	Type of interaction	From	To	Distance (Å)
R-MA	-4.3	Lysine292	Hydrogen bond	Lys292	*R*-MA	3.17
		Arginine290		*R*-MA	Arg290	2.78
		Proline391		*R*-MA	Pro391	2.23
		Lysine292	Electrostatic	Lys292	*R*-MA	4.83
		Arginine290	Hydrophobic	*R*-MA	Arg290	4.15
		Lysine292		*R*-MA	Lys292	5.22
S-MA	-4.6	Glutamine219	Hydrogen bond	Gln219	*S*-MA	3.01
				Gln219	*S*-MA	3.25
		Arginine290		*S*-MA	Arg290	2.45
		Proline391		*S*-MA	Pro391	2.49
		Arginine290	Hydrophobic	*S*-MA	Arg290	4.13
		Lysine292		*S*-MA	Lys292	5.48

Table B.9 - Docking affinity energy and interacting amino acids predicted by AutoDock Vina for Phycoerythrin- MA enantiomers.

Phycoerythrin (PDB:1EYX)	**Affinity (kcal.mol^{-1})**	**Interacting amino acids**	**Type of interaction**	**From**	**To**	**Distance (Å)**
R-MA	-4.3	Cysteine158	Hydrogen bond	Cys158	*R*-MA	3.67
		Aspartate39		*R*-MA	Asp39	3.61
		Lysine36	Hydrophobic	*R*-MA	Lys36	3.88
		Ileucine154		*R*-MA	Ile154	5.22
S-MA	-4.3	Serine102	Hydrogen bond	Ser102	*S*-MA	3.25
				Ser102	*S*-MA	2.83
		Aspartate101		*S*-MA	Asp101	2.87
				S-MA	Asp101	2.39
		Tyrosine168		Tyr168	*S*-MA	4.08
			Hydrophobic	Tyr168	*S*-MA	4.81

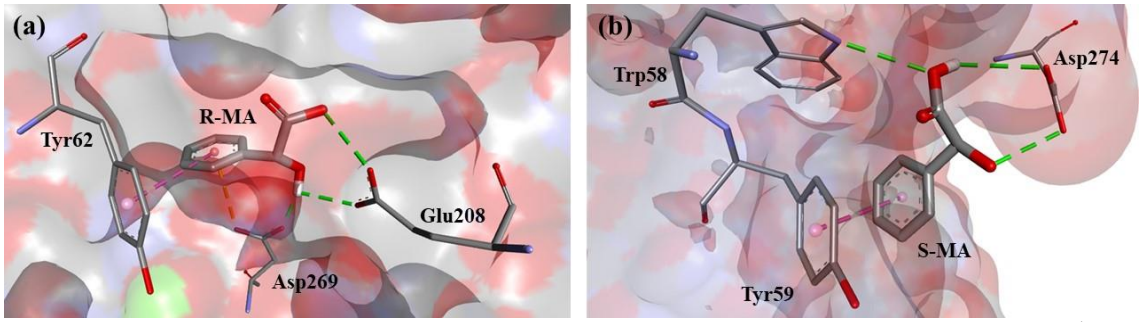

Figure B.1 - The Amylase docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for Amylase with (a) *R*-MA and (b) *S*-MA ((----) hydrogen bond, (----) π-anion bond and (----) π-alkyl bond.

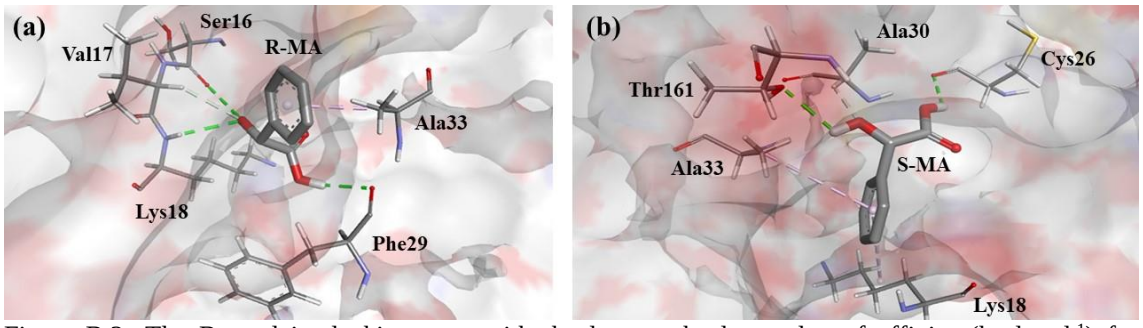

Figure B.2- The Bromelain docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for Bromelain with (a) *R*-MA and (b) *S*-MA ((----) hydrogen bond and (----) π-alkyl.

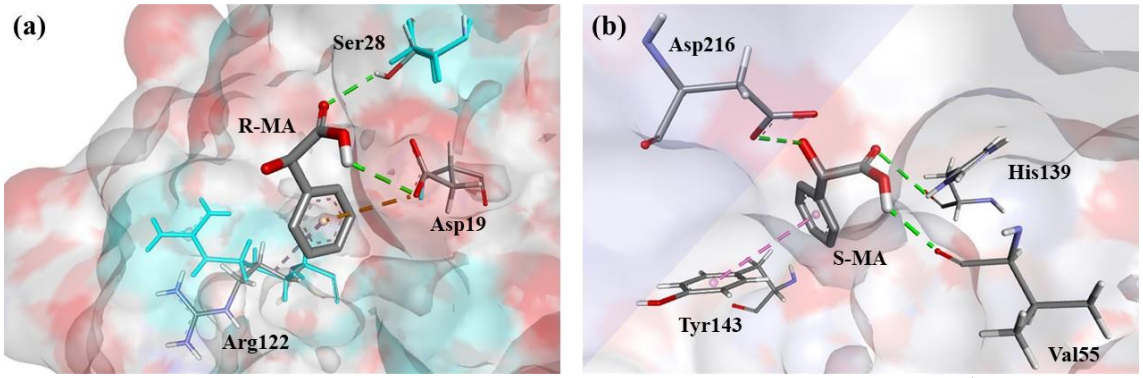

Figure B.3 - The GFP docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for GFP with (a) R-MA and (b) S-MA ((----) hydrogen bond, (----) π-anion bond, (----) π-alkyl bond and (----) π – π bond).

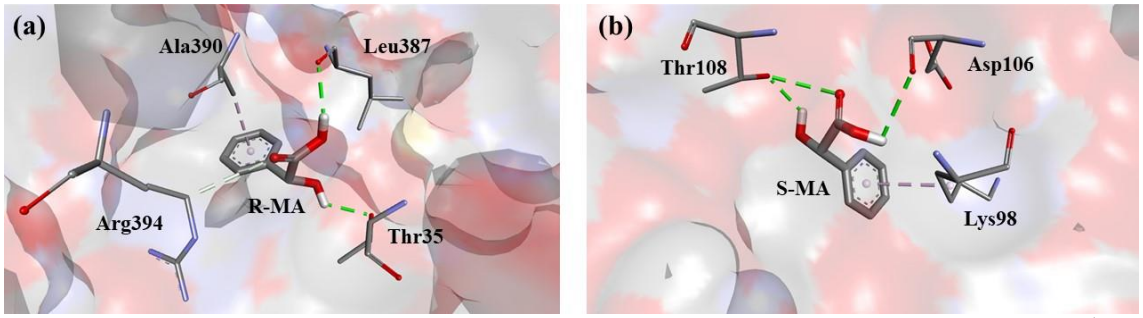

Figure B.4 - The Hexokinase PII docking pose with the lowest absolute value of affinity (kcal.mol^{-1})for Hexokinase PII with (a) *R*-MA and (b) *S*-MA ((----) hydrogen bond, (----) π-alkyl bond and (----) C-H bond).

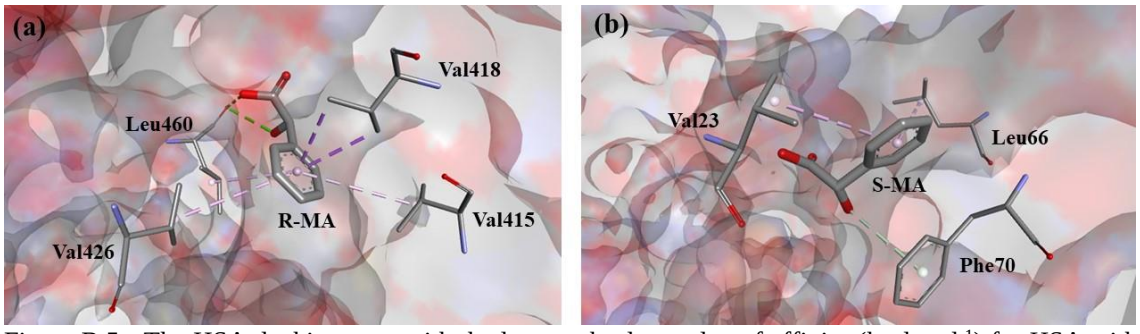

Figure B.5 - The HSA docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for HSA with (a) *R*-MA and (b) *S*-MA ((–––) hydrogen bond, (–––) π-σ bond and (–––) π-alkyl bond.

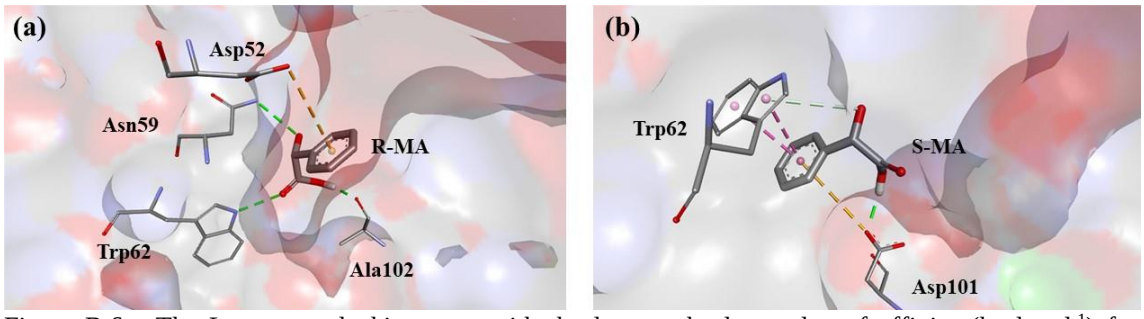

Figure B.6 - The Lysozyme docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for Lysozyme with (a) *R*-MA and (b) *S*-MA ((–––) hydrogen bond, (–––) π-σ bond, (–––) π-anion bond.

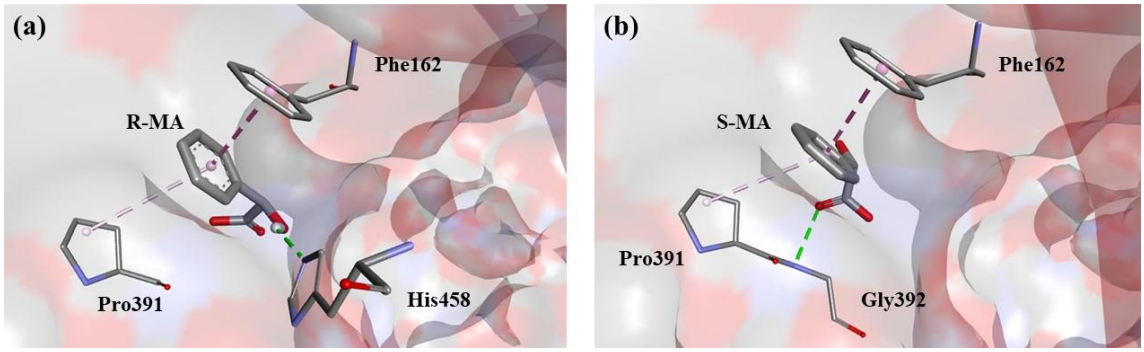

Figure B.7 - The Laccase docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for Laccase with (a) *R*-MA and (b) *S*-MA ((–––) hydrogen bond, (–––) π-alkyl bond and (–––) π – π bond).

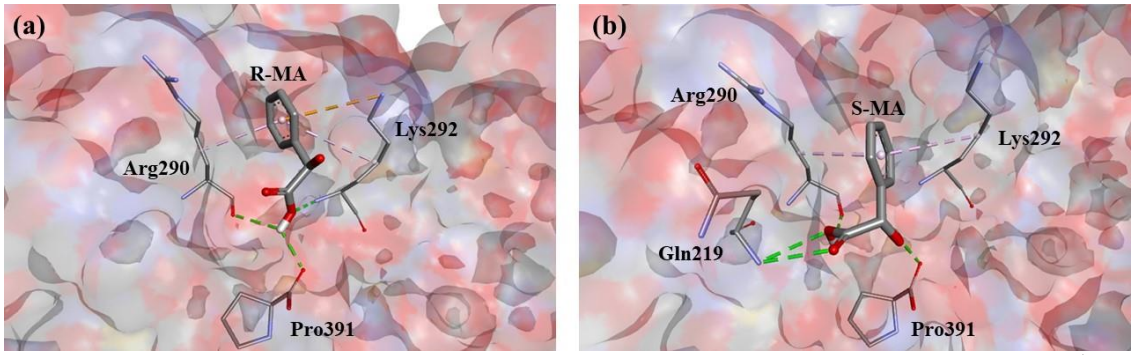

Figure B.8 - The Ovalbumin docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for Ovalbumin with (a) *R*-MA and (b) *S*-MA ((–––) hydrogen bond, (–––) π-anion bond and (–––) π-alkyl bond.

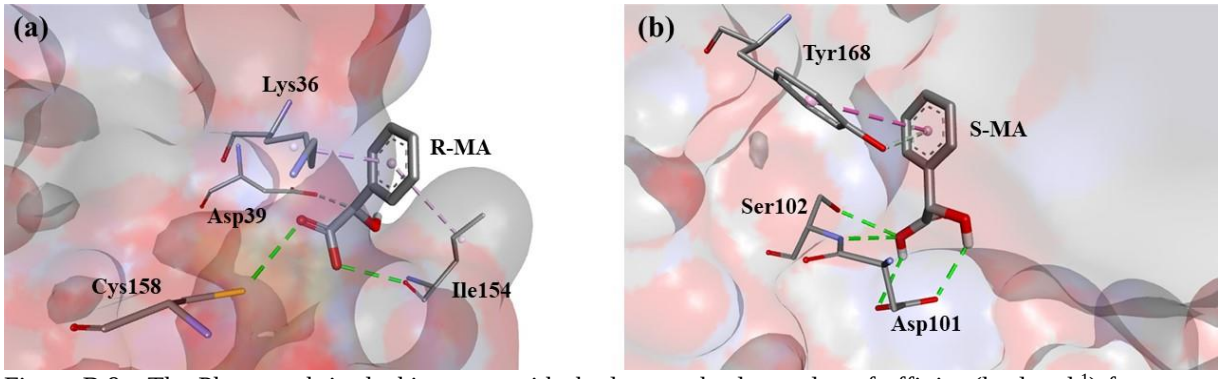

Figure B.9 - The Phycoerythrin docking pose with the lowest absolute value of affinity (kcal.mol^{-1}) for Phycoerythrin with (a) *R*-MA and (b) *S*-MA ((−−−−) hydrogen bond, (−−−−) π-alkyl bond and (−−−−) π – π bond).

C. Extraction efficiency and enantiomeric excess data

Table C.1 - Extraction efficiency of Cyt C ($E.E._{Cyt\ C}$), extraction efficiency and enantiomeric excess of MA in the presence and absence of Cyt C in the ABS studied.

Phase formers		**Mixture composition**		**MA (without Cyt C)**			**Cyt C**	**MA (with Cyt C)**		
1	**2**	**1 / (wt%)**	**2 / (wt%)**	**$E.E._{R\text{-}MA}$± σ / (%)**	**$E.E._{S\text{-}MA}$± / σ (%)**	**e.e. ± σ / (%)**	**$E.E._{cyt\ C}$± σ / (%)**	**$E.E._{R\text{-}MA}$± σ / (%)**	**$E.E._{S\text{-}MA}$± σ / (%)**	**e.e. ± σ / (%)**
PEG400	Na_2SO_4	22	12	2.94 ± 0.02	0.99 ± 0.13	1.2 ± 0.4	91.66 ± 0.22	2.76 ± 0.59	0.20 ± 0.80	1.2 ± 0.4
PPG400	[Ch][DHP]	30	30	43.44 ± 0.39	50.26 ± 0.91	-7.6 ± 0.5	86.13 ± 2.01	40.68 ± 3.14	48.69 ± 1.55	-9.0 ± 1.2
PEG400	PPG400	25	35	59.44 ± 1.62	73.82 ± 1.68	-9.8 ± 0.5	93.99 ± 0.50	64.95 ± 1.34	63.54 ± 1.61	1.9 ± 0.9
[C_4mim]Cl	$C_6H_5K_3O_7$	22	33	53.77 ± 4.56	55.05 ± 3.36	-1.3 ± 1.3	89.44 ± 0.75	91.68 ± 4.60	83.16 ± 3.07	5.0 ± 1.0
PEG3350	Dex500	5	15	40.69 ± 2.02	46.40 ± 1.56	-4.6 ± 0.9	70.74 ± 1.24	56.49 ± 3.17	49.84 ± 1.42	6.3 ± 1.6

Table C.2 - Extraction efficiency of BSA ($E.E._{BSA}$), extraction efficiency and enantiomeric excess of MA in the presence and absence of BSA in the ABS studied. Literature data extracted from Quental et al.[a], Taha et al.[b] and Pereira et al.[c]

Phase formers		**Mixture composition**		**MA (without BSA)**			**BSA**	**MA (with BSA)**		
1	**2**	**1 / (wt%)**	**2 / (wt%)**	**$E.E._{R\text{-}MA}$± σ / (%)**	**$E.E._{S\text{-}MA}$± σ / (%)**	**e.e. ± σ / (%)**	**$E.E._{BSA}$± σ / (%)**	**$E.E._{R\text{-}MA}$± σ / (%)**	**$E.E._{S\text{-}MA}$± σ / (%)**	**e.e. ± σ / (%)**
PPG400	[Ch][Ac]	30	30	99.45 ± 1.32	93.76 ± 3.62	3.2 ± 1.5	100[a]	87.80 ± 0.39	81.64 ± 0.74	3.8 ± 0.7
	[Ch][DHCit]	30	30	47.24 ± 1.49	52.08 ± 2.93	-4.6 ± 1.5	100[a]	46.42 ± 3.06	49.65 ± 3.91	-3.1 ± 0.6

	(D)-sucrose	30	30	26.47 ± 1.29	34.00 ± 1.45	-12.3 ± 0.5	100[b]	30.01 ± 0.18	33.68 ± 0.11	-5.9 ± 0.5
	Na_2SO_4	22	12	97.43 ± 2.42	86.90 ± 3.86	6.1 ± 1.9	55.00 ± 2.55	72.30 ± 1.31	69.71 ± 1.73	2.1 ± 0.6
	PEG400	35	25	59.44 ± 1.62	73.82 ± 1.68	-9.8 ± 0.5	90.36 ± 0.5	60.78 ± 2.83	64.71 ± 0.51	-3.0 ± 0.6
PEG3350	Dex500	5	15	40.69 ± 2.02	46.40 ± 1.56	-4.6 ± 0.9	89.43 ± 1.24	74.99 ± 1.10	76.29 ± 1.21	-1.0 ± 0.1
$[P_{i(444)1}][Tos]$		30	15	86.10 ± 3.30	81.74 ± 1.96	2.4 ± 0.8	100[c]	78.10 ± 1.97	71.44 ± 2.09	2.6 ± 1.6
$[P_{4444}]Cl$	$K_3C_6H_5O_7$/ $C_6H_8O_7$	30	15	89.00 ± 1.21	79.30 ± 0.85	5.6 ± 0.9	100[c]	83.07 ± 0.53	88.18 ± 1.47	2.1 ± 1.6
$[N_{4444}]Cl$		30	15	95.84 ± 2.21	91.18 ± 3.19	2.3 ± 0.8	100[c]	96.89 ± 2.31	86.56 ± 2.28	5.5 ± 1.2

Table C.3 - Extraction efficiency and enantiomeric excess of MA in the different systems with (D)-sucrose.

Phase formers		**Mixture composition (wt%)**				
1	**2**	**1 / (wt%)**	**2 / (wt%)**	**E.E.$_{R\text{-}MA}$± σ / (%)**	**E.E.$_{S\text{-}MA}$± σ / (%)**	**e.e. ± σ / (%)**
PPG400	(D)-Sucrose	26	26	41.42 ± 0.91	47.57 ± 0.74	-6.7 ± 1.7
		20	40	36.46 ± 0.69	45.30 ± 1.72	-10.6 ± 1.7
		30	30	26.47 ± 1.29	34.00 ± 1.45	-12.3 ± 0.5
$[C_4mim][CF_3SO_3]$		20	40	38.89 ± 1.42	47.82 ± 1.27	-10.6 ± 1.3